Safety Assessment of *Lactobacillus* Ferment Ingredients as Used in Cosmetics

Status:	Draft Report for Panel Review
Release Date:	May 16, 2025
Panel Meeting Date:	June 9 – 10, 2025

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Samuel M. Cohen, M.D., Ph.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume, M.B.A. This safety assessment was prepared by Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR.

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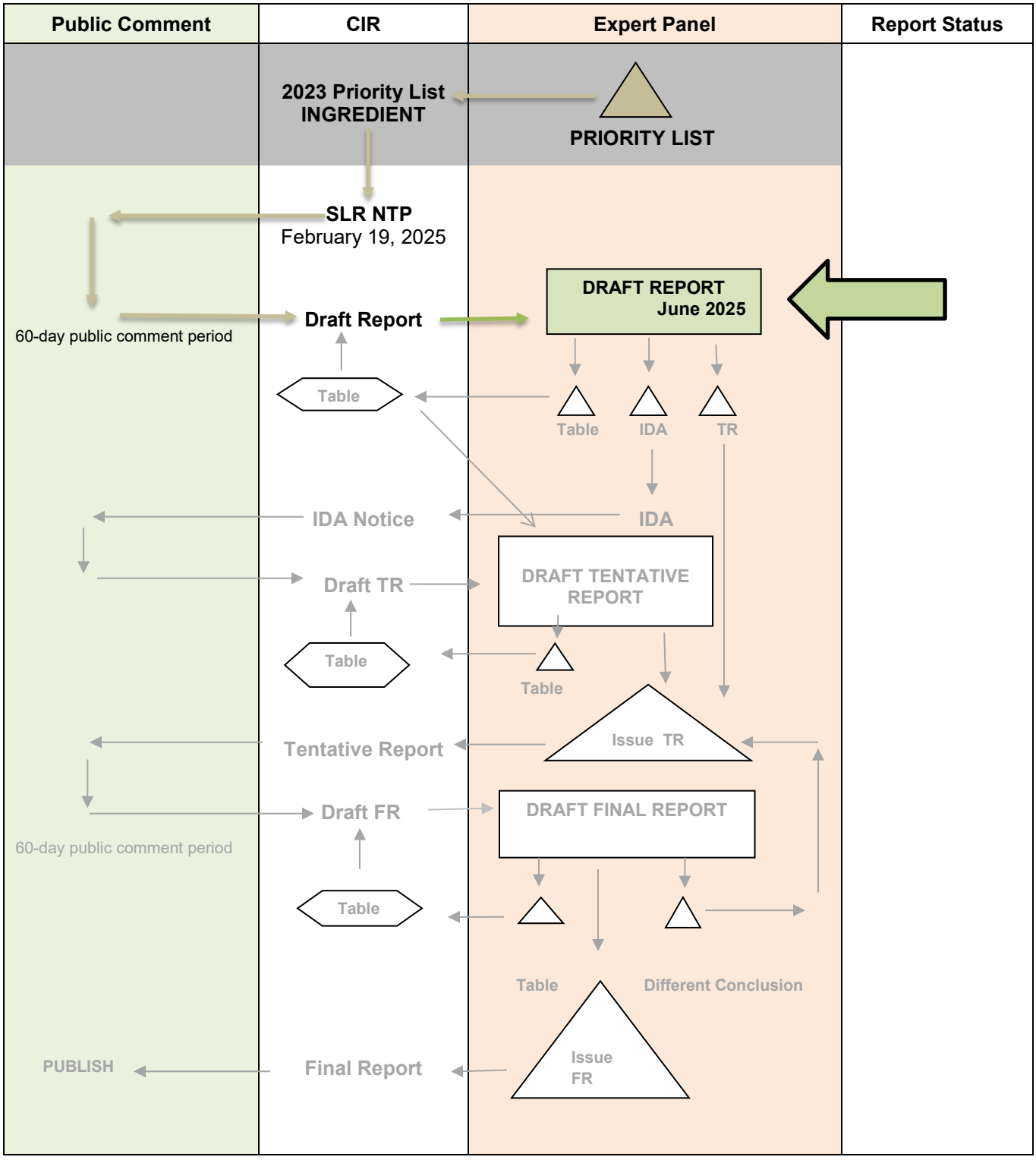
SAFETY ASSESSMENT FLOW CHART

INGREDIENT/FAMILY Lactobacillus Ferment Ingredients

MEETING June 2025



Cosmetic Ingredient Review

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Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Members and Liaisons
From: Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR
Date: May 16, 2025
Subject: Safety Assessment of Lactobacillus Ferment Ingredients

Enclosed is the Draft Report on the Safety Assessment of *Lactobacillus* Ferment Ingredients as Used in Cosmetics. (It is identified as *report_Lactobacillus_062025* in the pdf document). The four ingredients reviewed in this report include Lactobacillus Ferment, Lactobacillus Ferment Filtrate, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate.

A search was performed on the ingredients included in this grouping, as well as the species of *Lactobacilli* that are used in the manufacturing of these ingredients (as obtained from personal communication with the Personal Care Products Council (Council)). Ample data were found on these species as live bacteria (e.g., as probiotics in clinical studies, safety assessments, and case reports); however, these data were not included in the report as they are not relevant to the cosmetic ingredients reviewed herein. The main components of the ingredients reviewed in this report are not the live bacteria themselves, but byproducts of the fermentation of the bacteria (e.g., intracellular and extracellular metabolites, enzymes, peptides, teichoic acids, polysaccharides, organic acids (e.g., lactic acid), fragments of the dead bacteria). As there are many potential byproducts, the composition of these ingredients in cosmetics is unknown.

Due to the lack of data relevant to assessing the safety of these ingredients as used in cosmetics, a Notice to Proceed (NTP) Without the Preparation of a Scientific Literature Review was issued on November 21, 2024, requesting the following data:

- Composition (on products produced via the fermentation of relevant *Lactobacillus* species) and impurities data
- Method of manufacturing data
- Toxicokinetics data relevant to routes of exposure expected with cosmetic use
- General toxicity data
- Developmental and reproductive toxicity data
- Genotoxicity data
- Carcinogenicity data
- Dermal irritation and sensitization data
- Inhalation toxicity data
- Clarification on the species of *Lactobacilli* used in the production of these ingredients

Since the issuing of the NTP, new data have been submitted from industry. These data include:

- *data1_Lactobacillus_062025*
    - Composition information on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
    - Ames assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
    - Steroidogenesis assays on Lactobacillus Ferment and on a trade name mixture containing 2% Lactobacillus Ferment Lysate
    - In vitro dermal and ocular irritation assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate

  - In chemico and in vitro dermal sensitization assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
  - Human repeat insult patch tests (HRIPTs) on Lactobacillus Ferment
  - In vitro phototoxicity assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate

- *data2_Lactobacillus_062025*

  - Composition information on Lactobacillus Ferment Lysate Filtrate
  - HRIPT using a product containing 5% Lactobacillus Ferment Lysate Filtrate
  - In vitro dermal and ocular irritation assays on Lactobacillus Ferment Lysate Filtrate
  - Ames assay on Lactobacillus Ferment Lysate Filtrate

- *data3_Lactobacillus_062025*

  - Summary information regarding the composition of the Lactobacillus Ferment ingredients, including the species used in manufacturing
  - Manufacturing flow charts

While composition data were received on these ingredients, the byproducts found in the finished ingredients are still unknown. It should be noted that data on heat-killed *Lactobacillus* species were found in the literature; however, the relevancy of this data is unknown as these dead bacteria are assumed to be only a potential component of the finished ingredient. A sampling of these studies can be found in the Other Relevant Studies section of the report. ***The Panel should determine whether or not these studies should be kept in the report***.

Other information included in this packet include:

- concentration of use data (*data4_Lactobacillus_062025*)
- flow chart (*flow_ Lactobacillus_062025*)
- report history (*history_ Lactobacillus_062025*)
- search strategy (*search_ Lactobacillus_062025*)
- data profile (*dataprofile_ Lactobacillus_062025*)

If no further data are needed to reach a conclusion of safety, the Panel should formulate a Discussion and issue a Tentative Report. However, if additional data are required, the Panel should be prepared to identify those needs and issue an Insufficient Data Announcement.

Lactobacillus Ferment Ingredients – History

November 2024

-NTP issued requesting the following data:

- Composition (on products produced via the fermentation of relevant *Lactobacillus* species) and impurities data
- Method of manufacturing data
- Toxicokinetics data relevant to routes of exposure expected with cosmetic use
- General toxicity data
- Developmental and reproductive toxicity data
- Genotoxicity data
- Carcinogenicity data
- Dermal irritation and sensitization data
- Inhalation toxicity data
- Clarification on the species of *Lactobacilli* used in the production of these ingredients

April 2025

-Data received from industry:

- Composition information on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
- Ames assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
- Steroidogenesis assays on Lactobacillus Ferment and on a trade name mixture containing 2% Lactobacillus Ferment Lysate
- In vitro dermal and ocular irritation assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
- In chemico and in vitro dermal sensitization assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
- Human repeat insult patch tests (HRIPTs) on Lactobacillus Ferment
- In vitro photoxicity assays on Lactobacillus Ferment, trade name mixtures containing 2% Lactobacillus Ferment, a trade name mixture containing 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment, and a trade name mixture containing 2% Lactobacillus Ferment Lysate
- Composition information on Lactobacillus Ferment Lysate Filtrate
- HRIPT using a product containing 5% Lactobacillus Ferment Lysate Filtrate
- In vitro dermal and ocular irritation assays on Lactobacillus Ferment Lysate Filtrate
- Ames assay on Lactobacillus Ferment Lysate Filtrate
- Summary information regarding the composition of the Lactobacillus Ferment ingredients, including the species used in manufacturing
- Manufacturing flow charts

June 2025

-Panel reviews Draft Report

Lactobacillus Ferment Ingredients Data Profile* – June 2025 – Writer, Priya Ferguson																														
				Toxicokinetics			Acute Tox			Repeated Dose Tox			DART		Genotox		Carci		Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	Reported Use	Method of Mfg	Impurities	log P/log K_{ow}	Dermal Penetration	ADME	Dermal	Oral	Inhalation	Dermal	Oral	Inhalation	Dermal	Oral	In Vitro	In Vivo	Dermal	Oral	In Vitro	Animal	Human	In Vitro	Animal	Human	Phototoxicity	In Vitro	Animal	Single center	Case Reports
Lactobacillus Ferment	x	x	x												x				x			x		x	x	x		x	
Lactobacillus Ferment Filtrate	x	x																												
Lactobacillus Ferment Lysate	x	x													x				x		x	x			x	x			
Lactobacillus Ferment Lysate Filtrate	x	x	x												x				x			x		x		x			

* "x" indicates that data were available in a category for the ingredient

Lactobacillus Ferment Ingredients

Ingredient/species	CAS #	PubMed	FDA	HPVIS	NIOSH	NTIS	NTP	FEMA	EU	ECHA	ECETOC	SIDS	SCCS	AICIS	FAO	WHO	Web
Lactobacillus Ferment	2380025-37-4 (generic)	x							x								x
Lactobacillus Ferment Lysate	-	x							x								
Lactobacillus Ferment Lysate Filtrate	1686112-36-6								x								x
Lactobacillus Ferment Filtrate	-								x								

"x" noted if relevant information was found

Search Strategy

- INCI names searched in links provided below
- Species used in manufacturing searched along with the following key words
  - Heat
  - Heat-killed
  - Inactive
  - Dead
  - Cosmetic
- INCI names and species searched along with keywords in PubMed:
  - metabolism
  - dermal
  - inhalation
  - skin
  - toxicity
  - drugs
  - medicine
  - irritation
  - ocular
  - eye
  - sensitization
  - allergy
  - manufacture
  - cancer
  - probiotic
  - food
  - production
  - case report
  - cosmetic

LINKS

Search Engines

- Pubmed - http://www.ncbi.nlm.nih.gov/pubmed
- Connected Papers - https://www.connectedpapers.com/

Pertinent Websites

- wINCI - https://incipedia.personalcarecouncil.org/winci/ingredient-custom-search/
- FDA databases http://www.ecfr.gov/cgi-bin/ECFR?page=browse
- FDA search databases: http://www.fda.gov/ForIndustry/FDABasicsforIndustry/ucm234631.htm;,
- Substances Added to Food (formerly, EAFUS): https://www.fda.gov/food/food-additives-petitions/substances-added-food-formerly-eafus
- GRAS listing: http://www.fda.gov/food/ingredientspackaginglabeling/gras/default.htm
- SCOGS database: http://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm2006852.htm
- Indirect Food Additives: http://www.accessdata.fda.gov/scripts/fdcc/?set=IndirectAdditives
- Drug Approvals and Database: http://www.fda.gov/Drugs/InformationOnDrugs/default.htm
- FDA Orange Book: https://www.fda.gov/Drugs/InformationOnDrugs/ucm129662.htm
- (inactive ingredients approved for drugs: http://www.accessdata.fda.gov/scripts/cder/iig/
- HPVIS (EPA High-Production Volume Info Systems) - https://iaspub.epa.gov/oppthpv/public_search.html_page
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
  - technical reports search page: https://ntrl.ntis.gov/NTRL/
- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- Office of Dietary Supplements https://ods.od.nih.gov/
- FEMA (Flavor & Extract Manufacturers Association) GRAS: https://www.femaflavor.org/fema-gras
- EU CosIng database: http://ec.europa.eu/growth/tools-databases/cosing/
- ECHA (European Chemicals Agency – REACH dossiers) – http://echa.europa.eu/information-on-chemicals;jsessionid=A978100B4E4CC39C78C93A851EB3E3C7.live1
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- SCCS (Scientific Committee for Consumer Safety) opinions: http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/index_en.htm
- AICIS (Australian Industrial Chemicals Introduction Scheme)- https://www.industrialchemicals.gov.au/
- International Programme on Chemical Safety http://www.inchem.org/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) technical reports - http://www.who.int/biologicals/technical_report_series/en/
- www.google.com - a general Google search should be performed for additional background information, to identify references that are available, and for other general information

Botanical Websites, if applicable

- Dr. Duke's - https://phytochem.nal.usda.gov/phytochem/search
- Taxonomy database - http://www.ncbi.nlm.nih.gov/taxonomy
- GRIN (U.S. National Plant Germplasm System) - https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysimple.aspx
- Sigma Aldrich plant profiler- http://www.sigmaaldrich.com/life-science/nutrition-research/learning-center/plant-profiler.html
- American Herbal Products Association Botanical Safety Handbook (database) - http://www.ahpa.org/Resources/BotanicalSafetyHandbook.aspx
- National Agricultural Library NAL Catalog (AGRICOLA) https://agricola.nal.usda.gov/
- The Seasoning and Spice Association List of Culinary Herbs and Spices
- http://www.seasoningandspice.org.uk/ssa/background_culinary-herbs-spices.aspx

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ABBREVIATIONS

ARE	antioxidant/electrophile response element
BoSS	Burden of Sensitive Skin
CAS	Chemical Abstracts Service
CFU	colony-forming units
CIR	Cosmetic Ingredient Review
Council	Personal Care Products Council
Dictionary	*International Cosmetic Ingredient Dictionary and Handbook*
DPRA	direct peptide reactivity assay
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FOU	frequency of use
HRIPT	human repeated-insult patch test
l.o.	leave-on
MoCRA	Modernization of Cosmetics Regulation Act
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nrf2	nuclear factor erythroid 2-related factor 2
OECD	Organisation for Economic Co-operation and Development
OTC	over-the-counter
QPS	qualified presumption of safety
Panel	Expert Panel for Cosmetic Ingredient Safety
RLD	Registration and Listing Data
r.o.	rinse-off
SLS	sodium lauryl sulfate
TEWL	transepidermal water loss
TG	test guideline
US	United States
UVA	ultraviolet A
VCRP	Voluntary Cosmetic Registration Program

INTRODUCTION

This assessment reviews the safety of the following 4 ingredients as used in cosmetic formulations:

Lactobacillus Ferment
Lactobacillus Ferment Filtrate
Lactobacillus Ferment Lysate
Lactobacillus Ferment Lysate Filtrate

According to the web-based *International Cosmetic Ingredient Dictionary and Handbook (*wINCI *Dictionary)*, all of these *Lactobacillus* ferment ingredients are reported to function in cosmetics as skin-conditioning agents – miscellaneous; Lactobacillus Ferment is reported to have numerous other functions in cosmetics (Table 1).[1] These ingredients were grouped together as they are products of the fermentation of bacteria, predominantly from the genus *Lactobacillus.*

This safety assessment includes relevant published and unpublished data that are available for each endpoint that is evaluated. Published data are identified by conducting an extensive search of the world's literature; a search was last conducted April 2025. A listing of the search engines and websites that are used and the sources that are typically explored, as well as the endpoints that the Panel typically evaluates, is provided on the Cosmetic Ingredient Review (CIR) website (https://www.cir-safety.org/supplementaldoc/preliminary-search-engines-and-websites; https://www.cir-safety.org/supplementaldoc/cir-report-format-outline). Unpublished data are provided by the cosmetics industry, as well as by other interested parties.

The cosmetic ingredient names, according to the *Dictionary*, are written as listed above, without italics and without abbreviations. When referring to the bacterial species from which these ingredients are derived, the standard scientific practice of using italics will be followed (i.e., *Lactobacillus acidophilus*). If it is not known whether the substance being discussed is equivalent to the cosmetic ingredient, the test substance will be identified by the name used in the publication (e.g., *Lactobacillus* ferment lysate or *Lactobacillus acidophilus* ferment lysate) that is being cited. However, if it is known that the substance is a cosmetic ingredient, the *Dictionary* nomenclature (e.g., Lactobacillus Ferment or Lactobacillus Ferment derived from *Lactobacillus acidophilus*) will be used.

A search was performed on the ingredients and relevant bacterial species included in this grouping. Abundant data were found on *Lactobacilli* as live bacteria (e.g., as probiotics in clinical studies, safety assessments, and case reports); however, these data were not included herein as they are not relevant to the cosmetic ingredients reviewed. The main components of the ingredients reviewed in this report are not the live bacteria themselves, but byproducts of the fermentation of the bacteria (e.g., intracellular and extracellular metabolites, enzymes, peptides, teichoic acids, polysaccharides, organic acids (e.g., lactic acid), fragments of the dead bacteria).[2,3] As there are many potential byproducts, and the composition of these ingredients is unknown, clarification and information on composition is requested from industry.

In addition, a sampling of relevant studies found on heat-killed bacteria have been briefly summarized in the "Other Relevant Studies" section of this report. Details on these studies were omitted as these dead bacteria are assumed to be only a potential component of the finished ingredient (the amount of dead bacteria/bacterial fragments in finished ingredients is unknown).

CHEMISTRY

Definition

Lactobacillus Ferment is the product obtained through fermentation by the organism, *Lactobacillus*.[1] The definitions of the ingredients included in this review are provided in Table 1.

Chemical Properties

Lactobacillus Ferment and Lactobacillus Ferment Lysate Filtrate are liquids that is clear to yellow in color.[4,5] Other chemical properties on these two ingredients may be found in Table 2.

Bacteria Identification

According to personal communication with Personal Care Products Council and data from industry, several species of bacteria have been reported to be used in the manufacture of these *Lactobacillus* ferment ingredients. A listing of these species may be found in Table 3 The majority of the species of bacteria used in the preparation of these ingredients belong to the genus *Lactobacillus*.[6] Several of these species previously belonged to the *Lactobacillus* genus, but have recently been renamed. All listed species are non-spore-forming, gram-positive, lactic-acid producing bacteria, that are a normal part of the intestinal microbiota of various mammalian species.[7] The taxonomy of all of the species that have been reported to be used in the manufacturing of these ingredients may be found in Table 4.

Method of Manufacture

To manufacture *Lactobacillus* ferment-derived cosmetic ingredients, an appropriate growth medium is chosen and sterilized to ensure no unexpected microorganisms are present.[2] Bacteria are typically grown in modified media supplemented with determined substances (e.g., cysteine).[3] The *Lactobacillus* species is then inoculated into the media and incubated at an appropriate temperature for optimal growth. When the number of bacteria reaches a critical concentration,

the culture is harvested. If only intracellular content is desired, cells are harvested by centrifugation, and cell pellets are washed and then disrupted; washing and disruption are not performed if extracellular contents are desired.[3] Medium centrifugation is performed to remove intact cells and cellular debris. The products taken during this process (e.g., growth media, cellular content) to compose these ingredients is dependent upon ingredient type (described below). It should be noted that growth conditions, substrates, and manufacturing specifics may vary depending on the postbiotics of interest.

Manufacturing data on several trade name mixtures containing Lactobacillus Ferment, Lactobacillus Ferment Filtrate, and Lactobacillus Ferment Lysate were submitted by industry. These data may be found in Table 5.

Lactobacillus Ferment

According to a manufacturer, the fermentation process for Lactobacillus Ferment occurs in the presence of both standard growth media components and undecylenic acid derived from castor beans.[8] Secondary metabolites (e.g., water-soluble undecylenates) are formed as a stress response from the change in pH and oxygen levels for the bacteria. After fermentation, lysozyme is added to the culture to facilitate a controlled cell lysis to ensure the release of the antimicrobial peptides for maximized activity. Any existing lactic acid bacteria is removed via filtration.

Lactobacillus Ferment derived from *Lactobacillus acidophilus* is prepared by first testing for acceptance of the materials.[9,10] Following acceptance, fermentation of *Lactobacillus acidophilus* is performed in a defined media, under controlled conditions (pH, temperature, and time). Refiltration is then performed, followed by quality control, packing, sampling for micro, and shipping.

In the production of Lactobacillus Ferment, the whole culture is taken, and therefore the ingredient comprises both the microorganism and growth media.[2] The ferment is typically sterilized to kill the active bacteria.

Lactobacillus Ferment Filtrate

When preparing Lactobacillus Ferment Filtrate, following fermentation, the growth media and bacteria are filtered to separate the spent media from the bacterial cells.[2] The ingredient is composed of the conditioned media which contains the products that were excreted by the bacteria into the culture media.

Lactobacillus Ferment Lysate

In the preparation of Lactobacillus Ferment Lysate, the bacteria are broken open into the culture media.[2] The bacteria are lysed (and thus, killed), and the cellular contents are spilled into the media. The final ingredient contains the media containing these cellular contents, along with bacterial cell remainders (e.g., cell membranes, cell wall components, exopolysaccharides).

Lactobacillus Ferment Lysate Filtrate

Lactobacillus Ferment Lysate Filtrate is prepared by breaking open the bacteria into cultured media.[2] The resulting spilled cellular contents in the media, as well as the remainders of the lysed bacteria are then filtered and clarified.

Composition and Impurities

The *Lactobacillus* fermentation process produces postbiotics of interest including intracellular and extracellular metabolites, bacterial components, lactic acid, lipotechoic acid, and other organic acids through conversion of the sugars in the growth media, enzymes, and peptides.[2,3] Finished ingredients may contain all these components, including residual dead bacteria fragments, at varying amounts.

Lactobacillus Ferment

According to a manufacturer's specifications, Lactobacillus Ferment contains < 20 ppm heavy metals, < 10 ppm lead, < 2 ppm arsenic, and < 1 ppm cadmium, and may contain up to 10% water-soluble undecylenates.[11] In addition, none of the potential fragrance allergens listed in Annex III of European Union (EU) Cosmetic Regulation (EC) No. 1223/2009 or pesticides are present in this ingredient or in several trade name mixtures containing this ingredient.[12-17]

Lactobacillus Ferment derived from *Lactobacillus acidophilus* was determined to contain < 20 ppm heavy metals.[16] This ingredient was also reported to contain < 20 ppm chromium;, < 10 ppm lead, < 10 ppm nickel, < 10 ppm cobalt, < 5 ppm antimony, < 2 ppm arsenic, < 1 ppm mercury, and < 1 ppm cadmium. This ingredient may contain 1 – 10% bacteriocins.

Lactobacillus Ferment Lysate Filtrate

According to a manufacturer, Lactobacillus Ferment Lysate Filtrate is derived from *Lactobacillus bulgaricus* isolated from a yogurt starter culture and propagated on animal product-free media.[18] In addition, specifications indicate that this ingredient is 10 – 16% solids and contains < 20 ppm heavy metals, < 10 ppm lead, < 2 ppm arsenic, and < 1 ppm cadmium. The microbial content is reported to be < 100 colony-forming units (CFU)/g aerobic mesophiles, < 10 CFU/g anaerobic mesophiles, < 100 CFU/g mold and yeast, and no pathogens. None of the potential fragrance allergens listed in Annex III of EU/Cosmetic Regulation (EC) No. 1223/2009 or pesticides are present in this ingredient.[19]

Lactobacillus Ferment, Lactobacillus Ferment Filtrate, Lactobacillus Ferment Lysate

Composition information regarding trade name mixtures containing Lactobacillus Ferment, Lactobacillus Ferment Filtrate, and Lactobacillus Ferment Lysate were received. The majority of these mixtures contain Lactobacillus Ferment (97 – 99%) and 1,2-hexanediol (2 – 3%).[20] These data may be found in Table 6.

USE

Cosmetic

The safety of the cosmetic ingredients addressed in this assessment is evaluated based on data received from the US Food and Drug Administration (FDA) and the cosmetics industry on the expected use of *Lactobacillus* ferment ingredients in cosmetics. Data included herein were obtained from the FDA and in response to a survey of maximum use concentrations conducted by the Council, and it is these values that define the present practices of use and concentration. Frequencies of use obtained from the FDA include data from the Voluntary Cosmetic Registration Program (VCRP) database as well as Registration and Listing Data (RLD). As a result of the Modernization of Cosmetics Regulation Act (MoCRA) of 2022, the VCRP was discontinued in 2023 and, as of 2024, manufacturers and processors are required to register facilities and list their products (and ingredients therein) with the FDA (i.e., RLD). An exception is made for small businesses (average gross annual sales in the US of cosmetic products for the previous 3-year period is less than $1,000,000, adjusted for inflation), which are exempt from MoCRA reporting for most cosmetic product categories. However, to utilize the exemption, the small business must not cell eye area products, injected products, internal use products, or products that alter appearance for more than 24 h, and the facilities that manufacture these products, are not included in this exemption.[21] Please note, at this time, it is not appropriate to contrast data from the VCRP and RLD to determine a trend in frequency of use because there are numerous differences in the ways the data for the VCRP and the RLD were collected and processed, and because reporting frequency of use is now mandatory (as opposed to the past practice of voluntary reporting). Although the VCRP program is now defunct, trends in frequency of use from the RLD alone are not yet possible in that a baseline is currently not available.

According to 2023 VCRP data and 2024 RLD, Lactobacillus Ferment is reported to have the highest number of uses (it is reported be used in 266 and 2106 formulations, respectively; Table 7).[22,23] All other ingredients are reported to be used in 876 formulations or less (according to 2024 RLD). The results of the concentration of use survey conducted by the Council indicate Lactobacillus Ferment also has the highest concentration of use in a leave-on formulation; it is used at up to 1.5% in makeup bases.[24]

These ingredients are used in formulations that may result in incidental ingestion and ocular exposure as they are reported to be used in products applied near the mouth (e.g., Lactobacillus Ferment is used in lipsticks and lip glosses; concentration not reported) and near the eyes (e.g., Lactobacillus Ferment Lysate is used in eye lotions at up to 0.5%).[22-24] In addition, these ingredients are reported to be used in baby products (e.g., Lactobacillus Ferment is used in several baby products categories).

Additionally, some of the *Lactobacillus* ferment-derived ingredients are used in cosmetic powders and sprays and could possibly be inhaled; for example, Lactobacillus Ferment is reported to be used in cologne and toilet waters and face powders (concentrations not stated). In practice, as stated in the Panel's respiratory exposure resource document (https://www.cir-safety.org/cir-findings), most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and tracheobronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount. Conservative estimates of inhalation exposures to respirable particles during the use of loose powder cosmetic products are 400-fold to 1000-fold less than protective regulatory and guidance limits for inert airborne respirable particles in the workplace.

Some products containing these ingredients are marketed for use with airbrush delivery systems. With the advent of MoCRA and the current product categories outlined by the FDA, it is now mandatory that cosmetic products used in airbrush delivery systems be reported as such in the RLD. In other words, a reliable source of frequency of use data regarding the use of cosmetic ingredients in conjunction with airbrush delivery systems is now available in some instances. Some of the reported product categories for this ingredient as listed in the RLD do require designation if airbrush application is used, and this type of application was reported (e.g., Lactobacillus Ferment is reported to be used in foundations with airbrush application). Additionally, the Council currently surveys the cosmetic industry for maximum reported use concentrations of ingredients in products which may be used in conjunction with an airbrush delivery system; thus, this type of data may also be available when submitted. Please note that no concentration of use data were provided indicating airbrush application. Nevertheless, no consumer habits and practices data or particle size data are publicly available to evaluate the exposure associated with this use type, thereby preempting the ability to evaluate risk or safety. Without information regarding the consumer habits and practices data or product particle size data (or other relevant particle data, e.g., diameter) related to this use technology, the data profile is incomplete, and the Panel is not able to determine safety for use in airbrush formulations. Accordingly, the data are insufficient to evaluate the exposure resulting from cosmetics applied via airbrush delivery systems.

All of the *Lactobacillus* ferment ingredients named in the report are not restricted from use in any way under the rules governing cosmetic products in the European Union.[25]

Non-Cosmetic

The following information is on live bacterial strains; therefore, the relevance to the ingredients in this report is unknown.

According to 21CFR131.200, yogurt may be produced by culturing dairy ingredients with the lactic acid-producing bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus*. Prior sanctions were granted for the use of harmless lactic acid producing bacteria, such as *Lactobacillus acidophilus*, as optional ingredients in specified standardized foods.[26] These bacteria are permitted for use in cultured milk (which includes buttermilk) (21CFR131.112), sour cream (21CFR131.160), cottage cheese (21CFR133.128), and yogurt (21CFR131.200), provided that the mandatory cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* are also used in the yogurt. All of the bacterial species reported to be used in the production of the *Lactobacillus* ferment ingredients derived herein have a qualified presumption of safety (QPS) status, excluding *Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus kunkeei,* and *Lactobacillus gasseri*, as designated by the European Food Safety Authority (EFSA).[27]

In addition, all of the bacterial species (as listed in Table 3 and Table 4) are also found/used in common food products.[28-39] These products include, but are not limited to, yogurt, dairy products, fermented foods, and dietary supplements.

TOXICOKINETIC STUDIES

No relevant toxicokinetics studies on the *Lactobacillus* ferment-derived ingredients were found in the published literature, and unpublished data were not submitted.

TOXICOLOGICAL STUDIES

Acute and repeated-dose toxicity studies were not found in the published literature, and unpublished data were not submitted.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Developmental and reproductive toxicity studies were not found in the published literature, and unpublished data were not submitted.

GENOTOXICITY STUDIES

Details regarding the Ames assays summarized below may be found in Table 8.

Ames assays were performed with Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate, either as pure ingredients or as part of a trade name mixture.[40-47] The assays were performed in various strains for *Salmonella typhimurium* and with *Escherichia coli*, with and without metabolic activation, at concentrations up to 5000 µg/plate, and yielded negative results.

CARCINOGENICITY STUDIES

Carcinogenicity studies were not found in the published literature, and unpublished data were not submitted.

OTHER RELEVANT STUDIES

Endocrine Effects

Lactobacillus Ferment

An in vitro H295R steroidogenesis assay was performed in order to determine the potential of Lactobacillus Ferment derived from *Lactobacillus acidophilus* to induce or inhibit the production of 17β-estradiol and testosterone.[48] Human adrenocarcinoma H295R cells were exposed for 48 h to the test substance, diluted in dimethyl sulfoxide, at concentrations of 0.01, 0.1, 1, 10, 100, 1000, and 10,000 µM (each concentration evaluated in triplicate). Solvent and appropriate negative and positive controls were used. Following incubation, medium was removed, and cellular viability was analyzed. Concentrations of 17β-estradiol and testosterone were then measured, and absorbance values evaluated. The test substance was determined to be non-cytotoxic, and did not inhibit or induce the production of 17β-estradiol or testosterone. Control substances performed as expected.

Lactobacillus Ferment Lysate

An in vitro H295R steroidogenesis assay was performed according to the same procedure and concentrations stated above using a trade name mixture consisting of 2% Lactobacillus Ferment Lysate derived from *Lactobacillus acidophilus* and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract.[49] The test substance was determined to be non-cytotoxic, and did not inhibit or induce the production of 17β-estradiol or testosterone. Control substances performed as expected.

Effects on Pigmentation

Postbiotics obtained via fermentative processes of *Lactobacillus* bacteria, such as lactic acid, have been reported to result in skin depigmentation.[50] Other tyrosinase-inhibitors that may be present in these ingredients include cyclotetrapeptide, lipotechoic acid, daidzein, equol, uracil, and exo-polysaccharides (derived from *Lactobacillus sakei)*. These acids may be a component of the finished cosmetic ingredients reviewed in this report.

Lactobacillus Ferment Filtrate

A *Lactobacillus rhamnosus* spent-culture supernatant (heated for 30 min at 100°C) was evaluated for tyrosinase inhibitory activity in vitro.[51] An aqueous solution of mushroom tyrosinase was incubated with the test substance for 30 min. The amount of dopachrome produced in the reaction mixture was determined via spectrophotometric analysis, and percent inhibition of tyrosinase activity was calculated. Kojic acid (2 and 10 mM) was used as a standard. The test substance resulted in a 72.1 ± 1.2% inhibition of tyrosinase activity. (Inhibitory activities for 2 and 10 mM kojic acid were 43.6 ± 2.5 and 83.6 ± 1.0%, respectively.)

Effect on Damaged Skin

Lactobacillus Ferment Lysate

The effect of a cream containing *Lacticaseibacillus rhamnosus* ferment lysate on damaged skin was evaluated in 24 healthy female subjects.[52] Two sites of the skin were chosen on the forearms. Sites were treated with a 1% sodium lauryl sulfate (SLS) solution under occlusive conditions to induce skin damage. After a 24-h patch treatment with SLS, sites were evaluated, and subjects began application of the test substance to one site, 2x/d, for 5 d. (The second site was left untreated for comparison.) After 5-d, redness and drying by the SLS treatment were reduced in cream-treated sites compared to non-treated sites.

Heat-Killed Lactobacillus

Tyndallized *Lactobacillus acidophilus* has been observed to induce anti-melanogenesis via the reduction of mRNA expression of melanogenesis-related genes such as tyrosinase, tyrosinase-related protein 1, and tyrosinase-related protein 2.[53] Oral administration of heat-killed *Lactobacillus brevis* (0.05 or 0.5%) in mice via diet for 4 wk resulted in an inhibition of immunoglobin E production.[54] Heat-killed *Lacticaseibacillus paracasei* is reported to induce high levels of interleukin-12, resulting in immunomodulation, in studies performed in mice.[28] Oral administration (via diet; 55-d treatment) of heat-treated *Lactobacillus delbrueckii* subsp. *lactis* (0.08 and 0.33%) resulted in the prevention and alleviation of atopic dermatitis symptoms in mice.[55] In clinical studies, heat-killed *Lacticaseibacillus paracasei* was reported to potentiate immunity and prevent infection when orally administered to subjects (n = 45 – 241).[28,56] No adverse effects were reported in these studies. No adverse effects caused by the test substance were observed in a study in which 42 subjects were orally administered heat-killed *Lactobacillus brevis*.[57] Subjects were instructed to take either 25 or 50 mg of the test substance for 12 wk. Similarly, no adverse effects were observed in an assay in which subjects (n = 29) were orally given heat-killed *Lactobacillus helveticus* powder for 4 wk.[58]

DERMAL IRRITATION AND SENSITIZATION STUDIES

Details regarding the dermal irritation, sensitization, and phototoxicity studies summarized below may be found in Table 9.

EpiDerm™ assays were performed using Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate (either as a pure ingredient or as part of a trade name mixture).[59-65] All test substances were tested undiluted and were predicted to be non-irritating. No irritation was reported in a use assay in which 21 females applied cream containing *Lacticaseibacillus rhamnosus* lysate filtrate to the face, 2x/d, for 4 wk.[52]

The majority of these test substances were evaluated in direct peptide reactivity assays (DPRAs; at 100 mM) and antioxidant/electrophile response element (ARE)-nuclear factor erythroid 2-related factor (Nrf2) luciferase assays (at up to 2000 µM).[66-79] All test substances were predicted to be non-sensitizing. Human repeated-insult patch tests (HRIPTs) were performed using Lactobacillus Ferment in 2 assays (tested at 4 - 10%; 50 - 51 subjects) and using a product containing 5% Lactobacillus Ferment Lysate Filtrate (tested at 100%; 50 subjects).[80-82] All test substances were considered to be non-irritating and non-sensitizing.

Phototoxicity

In vitro phototoxicity assays were performed using Lactobacillus Ferment and Lactobacillus Ferment Lysate (either as pure ingredients or part of a trade name mixture).[83-86] The test substances were considered to be non-phototoxic. In vitro phototoxicity assays were also performed on Lactobacillus Ferment derived from *Lactobacillus acidophilus* (tested at 0.4, 1.3, 3.8, and 12%), Lactobacillus Ferment derived from *Lactobacillus acidophilus* (tested at 0.5, 1.5, 5, and 10%), and Lactobacillus Ferment (tested at 0.4, 1.2, 3.7, and 11%).[8,87,88] In these assays, no phototoxicity was observed at lower concentrations; however, at the highest concentrations tested, a decrease in cell viability was observed in non-irradiated and irradiated tissues (compared to 100% viability in non-irradiated and irradiated tissues in the negative control groups).

OCULAR IRRITATION STUDIES

Details regarding the in vitro ocular irritation studies summarized below may be found in Table 10.

EpiOcular™ assays were performed using Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate (either as pure ingredients or as part of a trade name mixture).[59-65] All test substances were tested at 100% and considered to be non-irritating.

CLINICAL STUDIES

Single Center Study

Lactobacillus Ferment

A redness assessment (evaluated using melanin measurement probes; redness profiles created using facial imaging system) and sensitivity self-assessment (Burden of Sensitive Skin (BoSS) questionnaire) was performed in 52 subjects with confirmed sensitive skin following use of a lotion containing a trade mixture consisting of fermented cultures of both *Lacticaseibacillus rhamnosus* and *Lactobacillus paracasei* (this mixture comprised 3% of the total lotion composition).[89] Lotion (0.6 – 0.8 g) was applied 2x/d, for 30 d. The mean value of skin redness decreased from 33.98 at day 0 to 29.3 at day 30 wasdetermined to be statistically significant compared to baseline ($p < 0.01$). The median skin redness profile values decreased from 12.79 to 11.08 after 30 d of treatment (compared to baseline; $p < 0.01$). Mean total BoSS questionnaire scores decreased from 23.29 to 17.63 after 30 d of treatment (compared to baseline; $p < 0.01$). Users reported decreased inflammation, scaling, itching, tingling, and pain following use of lotion.

The effect of topical application of postbiotic Himalaya-derived *Saccharomyces* and *Lactobacillus* ferment complex on the alleviation of sensitive scalp syndrome was evaluated in female volunteers ($n = 30$).[90] Subjects were treated with the ferment complex, 2x/d, for 4 wk. Symptoms were evaluated using a self-assessment questionnaire on day 0, 14, and 28. The self-assessment questionnaire revealed an overall syndrome alleviation effect of 100% after 14 and 28 d of treatment. The self-assessment demonstrated that all subjects felt alleviation of itching symptoms, 96% felt alleviation of prickling, tightness, pain, and dandruff symptoms, and 92% felt alleviation of burning symptoms.

SUMMARY

The safety of Lactobacillus Ferment, Lactobacillus Ferment Filtrate, Lactobacillus Ferment Lysate and Lactobacillus Ferment Lysate Filtrate is reviewed in this safety assessment. According to the *Dictionary*, all of these ingredients are mainly reported to function in cosmetics as skin-conditioning agents – miscellaneous. It should be noted that the main components of the ingredients reviewed in this report are not the live bacteria themselves, but byproducts of the fermentation of the bacteria (e.g., intracellular and extracellular metabolites, enzymes, peptides, teichoic acids, polysaccharides, organic acids (e.g., lactic acid), fragments of the dead bacteria).

According to 2023 FDA VCRP data and 2024 RLD, Lactobacillus Ferment is reported to have the highest number of uses (266 and 2106 formulations, respectively). Lactobacillus Ferment also has the highest concentration of use (it is used at up to 1.5% in makeup bases).

No genotoxicity was observed in Ames assays performed with Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate (either as a pure ingredient or as part of a trade name mixture). All test substances were evaluated with and without metabolic activation, at up to 5000 µg/plate.

In vitro H295R steroidogenesis assays were performed using a Lactobacillus Ferment derived from *Lactobacillus acidophilus* and a trade name mixture consisting of 2% Lactobacillus Ferment Lysate derived from *Lactobacillus acidophilus* and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract. Both test substances were tested at up to 10,000 µM, and neither test substance inhibited or induced the production of 17β-estradiol or testosterone.

Postbiotics obtained via a fermentative process of *Lactobacillus* bacteria (e.g., lactic acid, lipotechoic acid) are tyrosinase-inhibitors, and thus, may result in skin depigmentation. A heated *Lactobacillus rhamnosus* spent-culture supernatant resulted in a 72.1 ± 1.2% inhibition of tyrosinase activity in in vitro assay using mushroom tyrosinase. (The standard, 2 and 10 mM kojic acid, resulted in 43.6 ± 2.5 and 83.6 ± 1.0% tyrosinase inhibition, respectively).

A cream containing *Lacticaseibacillus rhamnosus* ferment lysate resulted in an alleviation of redness and drying induced by SLS, compared to sites untreated with cream. The cream was applied to the forearms of 24 female subjects 2x/d, for 5 d, after treatment with SLS.

Heat-killed *Lactobacillus* species have been reported to induce anti-melanogenesis in vitro, and inhibit immunoglobin E production, induce high levels of interleukin-12, and alleviate atopic dermatitis symptoms in mice. In humans, heat-killed *Lactobacillus* species were reported to potentiate immunity and prevent infection. No adverse effects were observed in studies in which humans were orally given heat-killed *Lactobacillus*.

EpiDerm™ assays were performed using Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate (either as a pure ingredient or as part of a trade name mixture). All test substances were tested

undiluted and were predicted to be non-irritating. No irritation was reported in a use assay in which 21 females applied cream containing *Lacticaseibacillus rhamnosus* lysate filtrate to the face, 2x/d, for 4 wk.

The majority of these test substances were evaluated in direct peptide reactivity assays (DPRAs; at 100 mM) and antioxidant/electrophile response element (ARE)-nuclear factor erythroid 2-related factor (Nrf2) luciferase assays (at up to 2000 µM). All test substances were predicted to be non-sensitizing. HRIPTs were performed using Lactobacillus Ferment in 2 assays (tested at 4 - 10%; 50 - 51 subjects) and using a product containing 5% Lactobacillus Ferment Lysate Filtrate (tested at 100%; 50 subjects). All test substances were considered to be non-irritating and non-sensitizing.

EpiOcular™ assays were performed using Lactobacillus Ferment, Lactobacillus Ferment Lysate, and Lactobacillus Ferment Lysate Filtrate (either as pure ingredients or as part of a trade name mixture). All test substances were tested at 100% and considered to be non-irritating.

Skin redness, skin redness profile values, and BoSS questionnaire scores were statistically significantly decreased in patients (n = 52) with sensitive skin following the use of a lotion containing a trade mixture consisting of fermented cultures of both *Lacticaseibacillus rhamnosus* and *Lactobacillus paracasei* (this mixture comprised 3% of the total lotion composition) 2x/d for 30 d. Alleviation of itching, prickling, tightness, dandruff, and burning was reported in female volunteers (n = 30) with sensitive scalp syndrome treated with a topical application of postbiotic *Saccharomyces* and *Lactobacillus* ferment complex.

DISCUSSION

To be developed.

CONCLUSION

To be determined.

TABLES

Table 1. Definitions, reported functions, and idealized structures of the ingredients in this safety assessment.[1]

Ingredient & CAS No.	Definition	Functions
Lactobacillus Ferment	Lactobacillus Ferment is the product obtained through fermentation by the microorganism, *Lactobacillus*	antiacne agents antidandruff agents antioxidants emulsion stabilizers film formers humectants skin-conditioning agents - miscellaneous viscosity increasing agents - aqueous
Lactobacillus Ferment Lysate	Lactobacillus Ferment Lysate is a lysate of the product obtained by the fermentation of *Lactobacillus*.	skin-conditioning agents - miscellaneous
Lactobacillus Ferment Lysate Filtrate CAS No. 1686112-36-6)	Lactobacillus Ferment Lysate Filtrate is a filtrate of the lysate of the product obtained by the fermentation of *Lactobacillus*.	skin-conditioning agents - miscellaneous
Lactobacillus Ferment Filtrate	Lactobacillus Ferment Filtrate is a filtrate of the product obtained by the fermentation of the growth media by the microorganism, *Lactobacillus*.	antioxidants skin-conditioning agents - miscellaneous

Table 2. Chemical properties

Property	Value	Reference
Lactobacillus Ferment		
Physical Form	liquid	4
Color	colorless - yellow	4
Odor	characteristic	4
pH	3.0 – 7.0	4
Specific Gravity (@ 25°C)	0.990 – 1.11	4
Boiling Point (°C)	100	8
Freezing Point (°C)	0	8
Lactobacillus Ferment Lysate Filtrate		
Physical Form	semi-viscous liquid	5
Color	clear to slightly hazy; yellow	5
Odor	characteristic	5
pH (@ 25°C)	3.5 – 5.5	5
Specific Gravity (@ 25°C)	1.02 – 1.07	5

Table 3. Species* used in the manufacturing of *Lactobacillus* ferment ingredients[6]

	Lactobacillus Ferment	Lactobacillus Ferment Filtrate	Lactobacillus Ferment Lysate	Lactobacillus Ferment Lysate Filtrate
Lactobacillus acidophilus	X		X	
Lactobacillus bifidus				X
Lactobacillus brevis	X		X	
Lactobacillus bulgaricus		X	X	X
Lactobacillus casei			X	
Lactobacillus crispatus	X	X		
Lactobacillus curvatus	X			
Lactobacillus delbrueckii			X	
Lactobacillus gasseri			X	
Lactobacillus helveticus			X	
Lactobacillus kunkeei	X			
Lactobacillus paracasei	X	X		
Lactobacillus pentosus	X			
Lactobacillus plantarum	X	X	X	
Lactobacillus reuteri	X			
Lactobacillus rhamnosus	X	X	X	
Lactobacillus reuteri		X		
Lactobacillus sakei			X	

*Some species listed herein are outdated species names. Table 4 lists the outdated names along with currently accepted names

Table 4. Taxonomy of species reported to be used in *Lactobacillus* ferment-derived cosmetic ingredients[91]

Class	Order	Family	Genus	Species
Bacilli	Lactobacillales	Lactobacillaceae	*Apilactobacillus*	*Apilactobacillus kunkeei* (previously known as *Lactobacillus kunkeei)*
Actinomycetes	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	*Bifidobacterium bifidum* (previously known as *Lactobacillus bifidus)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lacticaseibacillus*	*Lacticaseibacillus casei* (previously known as *Lactobacillus casei)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lacticaseibacillus*	*Lacticaseibacillus paracasei* (previously known as *Lactobacillus paracasei)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lacticaseibacillus*	*Lacticaseibacillus rhamnosus* (previously known as *Lactobacillus rhamnosus)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactiplantibacillus*	*Lactiplantibacillus pentosus* (previously known as *Lactobacillus pentosus)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactiplantibacillus*	*Lactiplantibacillus plantarum* (previously known as *Lactobacillus plantarum)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus acidophilus*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus bulgaricus* (synonymous to *Lactobacillus delbrueckii)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus crispatus*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus delbrueckii* (synonymous to *Lactobacillus bulgaricus)*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus gasseri*
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*Lactobacillus helveticus*
Bacilli	Lactobacillales	Lactobacillaceae	*Latilactobacillus*	*Latilactobacillus curvatus* (previously known as *Lactobacillus curvatus*)
Bacilli	Lactobacillales	Lactobacillaceae	*Latilactobacillus*	*Latilactobacillus sakei* (previously known as *Lactobacillus sakei)*
Bacilli	Lactobacillales	Lactobacillaceae	*Levilactobacillus*	*Levilactobacillus brevis* (previously known as *Lactobacillus brevis)*
Bacilli	Lactobacillales	Lactobacillaceae	*Limosilactobacillus*	*Limosilactobacillus reuteri*

Table 5. Method of manufacture of *Lactobacillus* ferment-derived ingredients[92-97]

Ingredient	Method	Manufacturing process
Lactobacillus Ferment	A	media preparation → sterilization and filtration → fermentation (addition of *Lactobacillus* sp.)→ sterilization and filtration → decolorization and deodorization → addition of other ingredients (as indicated in table 5 (e.g., 1,2-hexanediol)) → stabilization → quality control → packing
Lactobacillus Ferment	B	media preparation → media preparation → sterilization and filtration → fermentation (addition of *Lactobacillus plantarum*) → sterilization and filtration → addition of ethyl acetate and fraction → dehydration and concentration → addition of water and butylene glycol and dissolving → filtration → stabilization → quality control → packing
Lactobacillus Ferment	C	media preparation → sterilization and filtration → fermentation at 35°C for 48 h (addition of *Lactobacillus kunkeii*) → lysis and filtration → decolorization and deodorization → addition of 1,2-hexanediol → stabilization → quality control → packing
Lactobacillus Ferment	D	media preparation → sterilization and filtration → fermentation (addition of *Lactobacillus brevis*) → sterilization and filtration → decolorization and deodorization → addition of 1,2-hexanediol → stabilization → quality control → packing
Lactobacillus Ferment Filtrate	E	media preparation → sterilization and filtration → fermentation (addition of *Lactobacillus* sp.) → sterilization and filtration → decolorization and deodorization → addition of 1,2-hexanediol → filtration → stabilization → quality control → packing
Lactobacillus Ferment Lysate	F	media preparation (glucose, yeast extract, soy peptone) → sterilization → fermentation at 35°C for 72 h (addition of *Lactobacillus plantarum*) → quality control → tyndallization at 80°C for 1 h/d for 3 d → addition of 1,2-hexanediol → quality control → packing

*Some species listed herein are outdated species names. Table 4 lists the outdated names along with currently accepted names.

Table 6. Summary composition of *Lactobacillus* ferment-derived ingredients[20]

Ingredient	Species used in manufacturing*	Composition	Manufacturing method**
Lactobacillus Ferment	*Lactobacillus brevis*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus brevis*	97.8% Lactobacillus Ferment 2.2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus brevis*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus curvatus*	98% Lactobacillus Ferment 2% 1,2-hexanediol	E
Lactobacillus Ferment	*Lactobacillus curvatus*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus kunkeei*	98% Lactobacillus Ferment 2% 1,2-hexanediol	E
Lactobacillus Ferment	*Lactobacillus curvatus*	98% Lactobacillus Ferment 2% 1,2-hexanediol	C
Lactobacillus Ferment	*Lactobacillus paracasei*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus paracasei*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus pentosus*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus plantarum*	98% Lactobacillus Ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus plantarum*	98% Lactobacillus Ferment 2% 1,2-hexanediol	E
Lactobacillus Ferment	*Lactobacillus plantarum*	87.9% Lactobacillus Ferment 10% butylene glycol 2% 1,2-hexanediol 0.1% ethylhexylglycerin	A
Lactobacillus Ferment	*Lactobacillus plantarum*	97% Lactobacillus Ferment 3% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus plantarum*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus plantarum*	0.1% Lactobacillus Ferment 69.9% water 30% butylene glycol	B
Lactobacillus Ferment	*Lactobacillus plantarum*	99% Lactobacillus Ferment 0.8% propanediol 0.15% caprylyl glycol 0.05% ethylhexylglycerin	E
Lactobacillus Ferment	*Lactobacillus reuteri*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus rhamnosus*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	A
Lactobacillus Ferment	*Lactobacillus rhamnosus*	98% Lactobacillus ferment 2% 1,2-hexanediol	A
Lactobacillus Ferment Filtrate	*Lactobacillus plantarum*	98% Lactobacillus Ferment 2% 1,2-hexanediol	E

Table 6. Summary composition of *Lactobacillus* ferment-derived ingredients[20]

Ingredient	Species used in manufacturing*	Composition	Manufacturing method**
Lactobacillus Ferment Filtrate	*Lactobacillus reuteri*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	E
Lactobacillus Ferment Filtrate	*Lactobacillus rhamnosus*	97.8% Lactobacillus Ferment 2.2% 1,2-hexanediol	E
Lactobacillus Ferment Lysate	*Lactobacillus brevis*	97.5% Lactobacillus Ferment 2.5% 1,2-hexanediol	D
Lactobacillus Ferment Lysate	*Lactobacillus brevis*	98% Lactobacillus Ferment 2% 1,2-hexanediol	E
Lactobacillus Ferment Lysate	*Lactobacillus plantarum*	98% Lactobacillus Ferment 2% 1,2-hexanediol	F

*Some species listed herein are outdated species names. Table 4 lists the outdated names along with currently accepted names.
**Method of manufacture number correlates to the corresponding method in Table 5

Table 7. Frequency (RLD/VCRP) and concentration of use according to likely duration and exposure and by product category

	# of Uses		Max Conc of Use	# of Uses		Max Conc of Use
	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]
	Lactobacillus Ferment			Lactobacillus Ferment Filtrate		
Totals*	**2106**	**266**	**0.001 – 1.5**	**32**	**8**	**0.0005 – 0.09**
summarized by likely duration and exposure**						
Duration of Use						
Leave-On	***	*216*	*0.001 – 1.5*	***	*4*	*0.09*
Rinse-Off	***	*49*	*0.04*	***	*4*	*0.0005*
Diluted for (Bath) Use	***	*1*	*NR*	***	*NR*	*NR*
Exposure Type						
Eye Area	***	8	0.1	***	NR	NR
Incidental Ingestion	***	17	NR	***	NR	NR
Incidental Inhalation-Spray	***	1; 98[a]; 54[b]	0.2[a]	***	2[a]; 2[b]	NR
Incidental Inhalation-Powder	***	2; 54[b]; 1[c]	0.18[c]	***	2[b]	NR
Dermal Contact	***	233	0.01 – 1.5	***	8	0.0005 – 0.09
Deodorant (underarm)	***	NR	NR	***	NR	NR
Hair - Non-Coloring	***	16	0.001	***	NR	NR
Hair-Coloring	***	NR	NR	***	NR	NR
Nail	***	NR	NR	***	NR	NR
Mucous Membrane	***	32	NR	***	3	NR
Baby Products	***	1	NR	***	NR	NR
as reported by product category						
Baby Products	***6***					
Baby Shampoos	3	NR	NR			
Baby Lotions, Oils. Powders, Creams	2	1	NR			
Baby Wipes	1	NA	NR			
Other Baby Products						
Bath Preparations	***10***					
Other Bath Preparations	10	1	NR			
Eye Makeup Preparations (not children's)	***71***					
Eyeliner	2	1	NR			
Eye Shadow	1	NR	NR			
Eye Lotion	24	1	0.1			
Eye Makeup Remover	1	NR	NR			
Mascara	35	NR	NR			
Eyelash and Eyebrow Preparations (primers, conditioners, serums, fortifiers)	8	NA	NR			
Other Eye Makeup Preparations						
Fragrance Preparations	***25***					
Cologne and Toilet Water	19	NR	NR			
Perfumes	NR	1	NR			
Other Fragrance Preparation	6	NR	NR			
Hair Preparations (non-coloring)	***310***			***2***		
Hair Conditioners	20 (l.o.); 70 (r.o.)	**7**	NR			
Hair Sprays (aerosol fixatives)	10	NR	NR			
Permanent Waves	1	NR	NR			
Rinses (non-coloring)	13	1	NR			
Shampoos (non-coloring)	4 (l.o.); 76 (r.o.)	4	NR			
Tonics, Dressings, Other Hair Grooming Aids	32	3	NR	1	NR	NR
Wave Sets	2	NR	NR			
Other Hair Preparations	68 (l.o.); 30 (r.o.)	1	0.001	1 (r.o.)	NR	NR

Table 7. Frequency (RLD/VCRP) and concentration of use according to likely duration and exposure and by product category

	# of Uses		Max Conc of Use	# of Uses		Max Conc of Use
	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]
Hair Coloring Preparations	***81***					
Hair Dyes and Colors (all types requiring caution statements and patch tests)	80	NR	NR			
Hair Shampoos (coloring)	1 (r.o.)	NR	NR			
Other Hair Coloring Preparation						
Makeup Preparations (not eye or children's)	***186***					
Blushers and Rouges (all types)	12	NR	NR			
Face Powders	7	2	NR			
Foundations	111 (traditional application); 37 (airbrush application)	1	NR			
Lipstick and Lip Glosses	24	15	NR			
Makeup Bases	7 (traditional application); 2 (airbrush application)	2	1.5			
Makeup Fixatives	9	NR	NR			
Other Makeup Preparations	15 (l.o.); 6 (r.o.)	3	0.19			
Manicuring Preparations	***5***					
Basecoats and Undercoats	2	NR	NR			
Nail Polish and Enamel	3	NR	NR			
Oral Products	***11***					
Dentifrices	7	NR	NR			
Mouthwashes and Breath Fresheners	3	NR	NR			
Other Oral Products	1	2	NR			
Personal Cleanliness	***96***					
Bath Soaps and Body Washes	30	6	NR			
Deodorants (underarm)	41	NR	NR			
Douches						
Feminine Deodorants	NR	1	NR	NR	2	NR
Disposable Wipes	8	NA	NR			
Other Personal Cleanliness Products	5 (l.o.); 12 (r.o.)	7	NR	NR	1	NR
Shaving Preparations	***3***					
Beard Softeners	1	NR	NR			
Other Shaving Preparations	2	NR	NR			
Skin Care Preparations	***1310***			***30***		
Cleansing	127	15	0.04	3	3	0.0005
Face and Neck (excluding shaving preps)	737 (l.o.); 74 (r.o.)	49	0.18	24 (l.o.); 3 (r.o.)	NR	0.09
Body and Hand (excluding shaving preps)	66 (l.o.); 6 (r.o.)	4	NR	1 (l.o.)	NR	NR
Moisturizing	326	76	0.01 (not spray)	3	2	NR
Night	40	14	NR			
Paste Masks (mud packs)	41	7	NR			
Skin Fresheners	52	3	0.2	2	NR	NR
Other Skin Care Preparations	163 (l.o.); 74 (r.o.)	32	NR	4 (l.o.)	NR	NR
Suntan Preparations	***9***					
Suntan Gels, Creams, and Liquids	7	NR	NR			
Indoor Tanning Preparations	2 (spray application); 2 (professional spray application); 1 (airbrush application); 1 (professional airbrush application)	NR	NR			
Tattoo Preparations	***1***					
Other Tattoo Preparations	1	NA	NR			
Other Preparations (i.e., those preparations that do not fit another category)	***10***					

Table 7. Frequency (RLD/VCRP) and concentration of use according to likely duration and exposure and by product category

	# of Uses		Max Conc of Use	# of Uses		Max Conc of Use
	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]
	Lactobacillus Ferment Lysate			Lactobacillus Ferment Lysate Filtrate		
Totals*	**876**	**47**	**0.17 – 0.5**	**39**	**6**	**NR**
summarized by likely duration and exposure**						
Duration of Use						
Leave-On	***	*34*	*0.5*	***	*6*	*NR*
Rinse-Off	***	*13*	*0.17*	***	*NR*	*NR*
Diluted for (Bath) Use	***	*NR*	*NR*	***	*NR*	*NR*
Exposure Type						
Eye Area	***	3	0.5	***	NR	NR
Incidental Ingestion	***	1	NR	***	NR	NR
Incidental Inhalation-Spray	***	21[a]; 9[b]	NR	***	3[a]; 3[b]	NR
Incidental Inhalation-Powder	***	9[b]	NR	***	3[b]	NR
Dermal Contact	***	44	0.17 – 0.5	***	6	NR
Deodorant (underarm)	***	NR	NR	***	NR	NR
Hair - Non-Coloring	***	2	NR	***	NR	NR
Hair-Coloring	***	NR	NR	***	NR	NR
Nail	***	NR	NR	***	NR	NR
Mucous Membrane	***	4	NR	***	NR	NR
Baby Products	***	NR	NR	***	NR	NR
as reported by product category						
Baby Products	***9***					
Baby Shampoos	1	NR	NR			
Baby Lotions, Oils. Powders, Creams	5	NR	NR			
Baby Wipes						
Other Baby Products	3 (r.o.)	NR	NR			
Bath Preparations (diluted for use)						
Other Bath Preparations						
Eye Makeup Preparations	***13***					
Eyeliner						
Eye Shadow	1	NR	NR			
Eye Lotion	10	1	0.5			
Eye Makeup Remover						
Mascara						
Eyelash and Eyebrow Preparations (primers, conditioners, serums, fortifiers)	2	NA	NR			
Other Eye Makeup Preparations	NR	2	NR			
Fragrance Preparations						
Cologne and Toilet Water						
Perfumes						
Other Fragrance Preparation						
Hair Preparations (non-coloring)	***139***					
Hair Conditioners	6 (l.o.); 37 (r.o.)	NR	NR			
Hair Sprays (aerosol fixatives)	1	NR	NR			
Permanent Waves						
Rinses (non-coloring)	7	1	NR			
Shampoos (non-coloring)	64 (r.o.)	1	NR			
Tonics, Dressings, Other Hair Grooming Aids	15	NR	NR			
Wave Sets						
Other Hair Preparations	18 (l.o.); 8 (r.o.)	NR	NR			
Hair Coloring Preparations	***5***					
Hair Dyes and Colors (all types requiring caution statements and patch tests)	2	NR	NR			
Hair Shampoos (coloring)	1 (r.o.)	NR	NR			
Other Hair Coloring Preparation	2 (l.o.)	NR	NR			
Makeup Preparations (not eye or children's)	***39***					
Blushers and Rouges (all types)	2	NR	NR			
Face Powders						
Foundations	23 (traditional application)	NR	NR			
Lipstick and Lip Glosses	3	NR	NR			
Makeup Bases	4 (traditional application)	NR	NR			
Makeup Fixatives	3	NR	NR			
Other Makeup Preparations	4 (l.o.)	NR	NR			
Manicuring Preparations (Nail)						
Basecoats and Undercoats						
Nail Polish and Enamel						

Table 7. Frequency (RLD/VCRP) and concentration of use according to likely duration and exposure and by product category

	# of Uses		Max Conc of Use	# of Uses		Max Conc of Use
	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]	RLD (2024)[23]	VCRP (2023)[22]	% (2022)[24]
Oral Hygiene Products	***4***					
Dentifrices	3	NR	NR			
Mouthwashes and Breath Fresheners	1	1	NR			
Other Oral Products						
Personal Cleanliness Products	***99***					
Bath Soaps and Body Washes	11	NR	NR			
Deodorants (underarm)	1	NR	NR			
Douches	4	NR	NR			
Feminine Deodorants	3 (r.o.)	NR	NR			
Disposable Wipes	76	NA	NR			
Other Personal Cleanliness Products	1 (l.o.); 3 (r.o.)	3	NR			
Shaving Preparations						
Beard Softeners						
Other Shaving Preparations						
Skin Care Preparations	***575***			***38***		
Cleansing	59	6	NR	***2***	NR	NR
Face and Neck (excluding shaving preps)	353 (l.o.); 27 (r.o.)	9	NR	16 (l.o.); 2 (r.o.)	NR	NR
Body and Hand (excluding shaving preps)	29 (l.o.); 5 (r.o.)	NR	NR	2 (l.o.)	3	NR
Moisturizing	209	19	NR	15	1	NR
Night	7	NR	NR	1	2	NR
Paste Masks (mud packs)	9	1	0.17			
Skin Fresheners	19	NR	NR	6	NR	NR
Other Skin Care Preparations	37 (l.o.); 6 (r.o.)	2	NR	3 (l.o.)	NR	NR
Suntan Preparations	***2***					
Suntan Gels, Creams, and Liquids	2	NR	NR			
Indoor Tanning Preparations						
Tattoo Preparations						
Other Tattoo Preparations						
Other Preparations (i.e., those preparations that do not fit another category)	***6***			***1***		

NR – not reported; NA – not applicable (this category was not part of the VCRP)
l.o. – leave-on; r.o. – rinse-off
*The total FOU provided for RLD refers to the ingredient count supplied by FDA, and is not a summation of the number of uses per category because each product may be categorized under multiple product categories. For data supplied via the VCRP or by the Council survey, the sum of all exposure types may not equal the sum of total uses because each ingredient may be used in cosmetics with multiple exposure types.
**Likely duration and exposure are derived from VCRP and survey data based on product category (see Use Categorization https://www.cir-safety.org/cir-findings)
*** In the RLD each ingredient may be reported under several product categories, making a summation of RLD misleading in comparison to VCRP data. Accordingly, RLD are presented below by product category (as supplied by FDA), but are not summarized by likely duration and exposure.
[a] It is possible these products are sprays, but it is not specified whether the reported uses are sprays.
[b] Not specified whether a spray or a powder, but it is possible the use can be as a spray or a powder, therefore the information is captured in both categories
[c] It is possible these products are powders, but it is not specified whether the reported uses are powders.

Table 8. In vitro genotoxicity studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test System	Protocol	Results	Reference
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lacto-bacillus acidophilus*, 48% water, 30% glycerin, and 20% pereskia aculeata callus extract	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; sterile water used as negative control; a known mutagen used as positive control	non-genotoxic; controls performed as expected	[43]
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 20% momordica cochinchinesis seed extract, and 78% water	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[45]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lacto-bacillus acidophilus*	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[41]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lacto-bacillus acidophilus*	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	. *typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[47]
Lactobacillus Ferment	Lactobacillus Ferment	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[42]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate (derived from *Lactobacillus acidophilus*) and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[40]
Lactobacillus Ferment Lysate Filtrate	Lactobacillus Ferment Lysate Filtrate derived from *Lactobacillus bifidus* (98% purity)	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2*uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[46]

Table 8. In vitro genotoxicity studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test System	Protocol	Results	Reference
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% camelia sinensis leaf extract, 10% punica granatum extract, and 1% caffeine *Lactobacillus* species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	distilled water	1.5, 5, 15, 50, 150, 500, 1500, 5000 µg/plate	*S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP*2uvrA*	OECD TG 471; Ames assay performed with and without metabolic activation; as described above	non-genotoxic; controls performed as expected	[44]

OECD = Organisation for Economic Cooperation and Development; TG = test guidelines

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
				IRRITATION			
				IN VITRO			
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 20% *Momordica cochinchinesis* seed extract, and 78% water	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay; cell viability evaluated using MTT assay; phosphate-buffered saline used as negative control; sodium dodecyl sulfate used as positive control	predicted to be non-irritating; controls gave expected results	[60]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[64]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[65]
Lactobacillus Ferment	Lactobacillus Ferment	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[63]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate (derived from *Lactobacillus acidophilus*) and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[62]
Lactobacillus Ferment Lysate Filtrate	Lactobacillus Ferment Lysate Filtrate derived from *Lactobacillus bifidus* (98% purity)	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[59]

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% *Camelia sinensis* leaf extract, 10% *Punica granatum* extract, and 1% caffeine Lactobacillus species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	none	100%; 30 µl	reconstructed human epidermis (n = 3)	EpiDerm™ assay. as described above	predicted to be non-irritating; controls gave expected results	[61]
				HUMAN			
Lactobacillus Ferment Lysate	cream containing *Lacticaseibacillus rhamnosus* ferment lysate (composition of cream not stated)	none	100%	21 female subjects	use study; subjects instructed to apply cream to face 2x/d for 4 wk	no erythema, edema, stinging, burning, tingling, or tightness reported	[52]
				SENSITIZATION			
				IN CHEMICO/IN VITRO			
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 48% water, 30% glycerin, and 20% *Pereskia aculeata* callus extract	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; negative control: dimethyl sulfoxide; positive control: cinnamic aldehyde	predicted to be non-sensitizing; reference control gave expected results	[70]
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment, 20% *Momordica cochinchinesis* seed extract, and 78% water	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[72]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[67]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[66]
Lactobacillus Ferment	Lactobacillus Ferment	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[68]
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 48% water, 30% glycerin, and 20% *Pereskia aculeata* callus extract	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; solvent control; cinnamic aldehyde as positive control	predicted to be non-sensitizing; controls gave expected results	[77]
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 20% *Momordica cochinchinesis* seed extract, and 78% water	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[79]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[74]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[73]

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
Lactobacillus Ferment	Lactobacillus Ferment	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[75]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate (derived from *Lactobacillus acidophilus*) and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[69]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate (derived from *Lactobacillus acidophilus*) and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[76]
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% camelia sinensis leaf extract, 10% punica granatum extract, and 1% caffeine Lactobacillus species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	acetonitrile	100 mM	cysteine and lysine peptides	OECD TG 442C; DPRA; as described above	predicted to be non-sensitizing; reference control gave expected results	[71]
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% camelia sinensis leaf extract, 10% punica granatum extract, and 1% caffeine Lactobacillus species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	dimethyl sulfoxide	0.98 – 2000 µM	KeratinoSens™ tissues; 50 µl	OECD TG 442D; ARE-Nrf2 luciferase assay; as described above	predicted to be non-sensitizing; controls gave expected results	[78]

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
				HUMAN			
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	distilled water	4%; 0.2 ml	50 subjects	HRIPT; test material applied under occlusive patches placed directly on the skin of the infrascapular region of the back; patch removed after 24 h; procedure repeated 3x/wk for 3 wk until a series of 9 consecutive 24 h applications were made ; following a 10 – 14 d non-treatment period; a challenge patch was applied to previously unexposed site; reactions scored 24 and 48 h after application	non-irritating and non-sensitizing	[82]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	distilled water	10%; 0.2 ml	51 subjects	HRIPT performed under occlusive conditions according to the same procedures as stated above	non-irritating and non-sensitizing 9 d after study initiation, one subject had an allergic reaction on the face that was treated with methylprednisolone; investigator discontinued this subject's participation, and judged that it was unlikely that the reaction was related to the test material	[80]
Lactobacillus Ferment Lysate Filtrate	product containing 5% Lactobacillus Ferment Lysate Filtrate derived from *Lactobacillus bulgaricus* (purity 99.1%)	NR	100%; 0.2 ml	50 subjects	HRIPT performed under occlusive conditions according to the same procedures as stated above	non-irritating and non-sensitizing	[81]
				PHOTOSENSITIZATION			
				IN VITRO			
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 48% water, 30% glycerin, and 20% *Pereskia aculeata* callus extract	NR	0.5, 1.5, 5, and 10%	reconstructed human epidermis tissues (n = 2/concentration)	phototoxicity assay; tissue inserts incubated with test substance or negative (sterile deionized water) or positive (chloropromazine) controlst; appropriate tissues were irradiated with UVA (6 J/cm2) for 60 min (some tissues left un-irradiated for comparison); after incubation and irradiation, cell viability was evaluated in MTT assay	non-phototoxic; controls gave expected results	[83]
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 20% *Momordica cochinchinesis* seed extract, and 78% water	NR	0.5, 1.5, 5, and 10%	reconstructed human epidermis tissues (n = 2/concentration)	phototoxicity assay performed according to the same procedures as above	non-phototoxic; controls gave expected results	[86]

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	NR	0.4, 1.3, 3.8, and 12%	reconstructed human epidermis tissues (n = 2/concentration)	phototoxicity assay performed according to the same procedures as above	test substance considered to be non-phototoxic at concentrations of 0.4, 1.3, and 3.8%; a decrease in viability at the 12% test concentration was observed with and without irradiation (tissue viability was approximately 70 and 50% in non-irradiated and irradiated tissues, respectively, compared to 100% viability in the negative control group); negative and positive controls performed as expected	[88]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*		0.5, 1.5, 5, and 10%	reconstructed human epidermis tissues (n = 2/ concentration)	phototoxicity assay performed according to the same procedures as above	test substance considered to be non-phototoxic at concentrations of 0.5, 1.5, and 5%; a decrease in viability at the 10% concentration was observed with and without irradiation (tissue viability was approximately 85 and 80% in non-irradiated and irradiated tissues, respectively, compared to 100% viability in non-irradiated and irradiated tissues in the negative control group); negative and positive controls performed as expected	[87]
Lactobacillus Ferment	Lactobacillus Ferment	NR	0.4, 1.2, 3.7, and 11%	reconstructed human epidermis tissues (n = 2/ concentration)	phototoxicity assay performed according to the same procedures as above	test substance was considered to be non-phototoxic at concentrations of 0.4, 1.2, and 3.7%; a decrease in cell viability at the 11% concentration was observed with and without irradiation (tissue viability was approximately 70 and 60% in non-irradiated and irradiated tissues, respectively, compared to 100% viability in both non-irradiated and irradiated tissues in the negative control group); negative and positive controls performed as expected	[8]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate (derived from *Lactobacillus acidophilus*) and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	NR	0.5, 1.5, 5, and 10%	reconstructed human epidermis tissues (n = 2/ concentration)	phototoxicity assay performed according to the same procedures as above	non-phototoxic; controls gave expected results	[84]

Table 9. Dermal irritation and sensitization studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population/System	Protocol	Results	Reference
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% *Camelia sinensis* leaf extract, 10% *Punica granatum* extract, and 1% caffeine *Lactobacillus* species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	NR	0.5, 1.5, 5, and 10%	reconstructed human epidermis tissues (n = 2/ concentration)	phototoxicity assay performed according to the same procedures as above	non-phototoxic; controls gave expected results	[85]

ARE = antioxidant/electrophile response element; DPRA = direct peptide reactivity assay; HRIPT = human repeat insult patch test; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NR = not reported; Nrf2 = nuclear factor erythroid 2-related factor 2; OECD = Organisation for Economic Cooperation and Development; TG = test guideline; UVA = ultraviolet A

Table 10. In vitro ocular irritation studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population	Protocol	Results	Reference
			IN VITRO				
Lactobacillus Ferment	trade name mixture consisting of 2% Lactobacillus Ferment derived from *Lactobacillus acidophilus*, 20% *Momordica cochinchinesis* seed extract, and 78% water	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; 90 min incubation; cell viability evaluated via MTT assay; sterilized deionized water as negative control; methyl acetate as positive control	non-irritating; controls gave expected results	[60]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[64]
Lactobacillus Ferment	Lactobacillus Ferment derived from *Lactobacillus acidophilus*	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[65]
Lactobacillus Ferment	Lactobacillus Ferment	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[63]
Lactobacillus Ferment Lysate	trade name mixture consisting of 2% Lactobacillus Ferment Lysate derived from *Lactobacillus acidophilus* and 98% *Medicago sativa* (alfalfa) callus culture conditioned media extract	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[62]
Lactobacillus Ferment Lysate Filtrate	Lactobacillus Ferment Lysate Filtrate derived from *Lactobacillus bifidus* (98% purity)	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[59]

Table 10. In vitro ocular irritation studies

Ingredient	Test Article	Vehicle	Concentration/Dose	Test Population	Protocol	Results	Reference
Lactobacillus Ferment and Lactobacillus Ferment Lysate	trade name mixture consisting of 10% Lactobacillus Ferment Lysate, 2% Lactobacillus Ferment, 67% water, 10% *Camelia sinensis* leaf extract, 10% *Punica granatum* extract, and 1% caffeine Lactobacillus species used in manufacturing of Lactobacillus Ferment ingredients in this trade name mixture include *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (unknown which species corresponds to the ferment or ferment lysate)	none	100%; 50 µl	cornea epithelial model (n = 2)	EpiOcular™ assay; as described above	non-irritating; controls gave expected results	[61]

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; OECD = Organisation for Economic Cooperation and Development; TG = test guidelines

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Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: April 21, 2025

SUBJECT: Lactobacillus Ferment Ingredients

Active Concepts LLC. 2024. Compositional Breakdown AC Baby Jackharides (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*)).

Active Concepts LLC. 2022. Dermal and Ocular Irritation Tests AC Baby Jackharides (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*).

Active Concepts LLC. 2022. In Chemico Skin Sensitization AC Baby Jackharides (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*).

Active Concepts LLC. 2022. OECD TG 442D: In Vitro Skin Sensitization (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*).

Active Concepts LLC. 2022. Phototoxicity Assay Analysis. (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*).

Active Concepts LLC. 2022. Bacterial Reverse Mutation Assay. (contains 2% Lactobacillus Ferment (*Lactobacillus acidophilus*).

Active Concepts LLC. 2023. Compositional Breakdown Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment (*Lactobacillus bulgaricus* and *Lactobacillus acidophilus*)).

Active Concepts LLC. 2021. Dermal and Ocular Irritation Tests Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment).

Active Concepts LLC. 2021. In Chemico Skin Sensitization Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment).

Active Concepts. 2021. In Vitro Skin Sensitization Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment).

Active Concepts. 2021. Phototoxicity Assay Analysis Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment).

Active Concepts. 2021. Bacterial Reverse Mutation Test Revital-Eyes (contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment).

Active Concepts. 2025. Compositional Breakdown AC Barbados Gooseberry Extract G SF (contains 2% Lactobacillus Ferment).

Active Concepts. 2022. Phototoxicity Assay Analysis AC Barbados Gooseberry Extract G SF (contains 2% Lactobacillus Ferment).

Active Concepts. 2023. Bacterial Reverse Mutation Assay Barbados Gooseberry Extract G SF (contains 2% Lactobacillus Ferment).

Active Concepts. 2022. OECD 442C: In Chemico Skin Sensitization Barbados Gooseberry Extract G SF (contains 2% Lactobacillus Ferment).

Active Concepts. 2022. OECD 442D: In Vitro Skin Sensitization Barbados Gooseberry Extract G SF (contains 2% Lactobacillus Ferment).

Active Concepts. 2023. Compositional Breakdown AC AlfalfaBoost (contains 2% Lactobacillus Ferment Lysate).

Active Concepts. 2023. Dermal and Ocular Irritation Tests AlfalfaBoost (contains 2% Lactobacillus Ferment Lysate).

Active Concepts. 2023. Phototoxicity Assay Analysis AlfalfaBoost (contains 2% Lactobacillus Ferment Lysate).

Active Concepts. 2023. OECD 442D: In vitro skin sensitization AlfalfaBoost (contains 2% Lactobacillus Ferment Lysate).

Active Concepts. 2023. OECD 442C: In Chemico skin sensitization AlfalfaBoost (contains 2% Lactobacillus Ferment Lysate).

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Active Micro Technologies. 2019. Repeat Insult Patch Test Skin Irritation / Sensitization Evaluation (Occlusive Patch) Leucidal® SF Max.

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Active Micro Technologies. 2018. Dermal and Ocular Irritation Tests Leucidal® SF Max.

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AC Baby Jackharides
Code: 20963CHI

Compositional Breakdown:

Ingredient	%
Water	78.00
Momordica Cochinchinensis Seed Extract	20.00
Lactobacillus Ferment	2.00

Made with Lactobacillus acidophilus
Suggested use level: 1-10%

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Dermal and Ocular Irritation Tests

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Tradename: AC Baby Jackharides contains 2% Lactobacillus Ferment

Code: 20963CHI

CAS #: 7732-18-5 & 93333-78-9 (or) 94094-86-7 & 68333-16-4 (or) 1686112-36-6 (or) 92128-81-9

Test Request Form #: 9434

Lot #: N210504I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

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 **AC Baby Jackharides** 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 **non-irritating**. 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% CO_2, 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-irritant**. The negative and positive controls performed as anticipated.

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Dermal and Ocular Irritation Tests

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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.

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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 Concepts, 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% CO_2 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% CO_2 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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

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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 Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

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Dermal and Ocular Irritation Tests

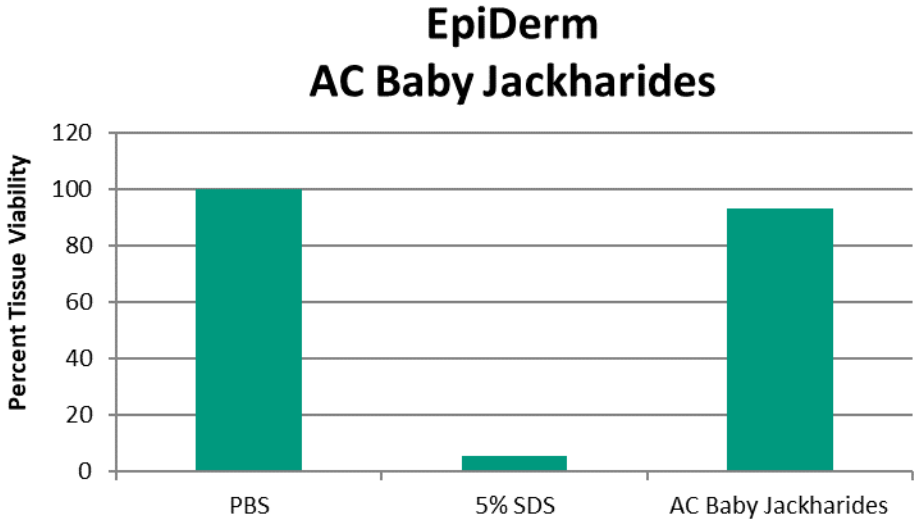

Figure 1: EpiDerm tissue viability

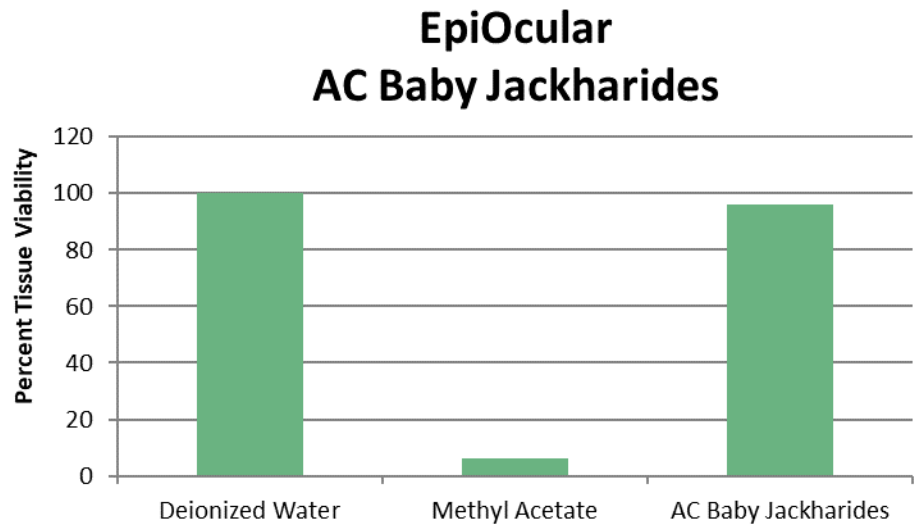

Figure 2: EpiOcular tissue viability

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: AC Baby Jackharides contains 2% Lactobacillus Ferment

Code: 20963CHI

CAS #: 7732-18-5 & 93333-78-9 (or) 94094-86-7 & 68333-16-4 (or) 1686112-36-6 (or) 92128-81-9

Test Request Form #: 8043

Lot #: N210504I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

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 contact[1]. 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 dermatitis[2]. 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 **AC Baby Jackharides** 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.

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OECD TG 442C: *In Chemico* Skin Sensitization

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-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-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* **AC Baby Jackharides** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **AC Baby Jackharides**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

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
• 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)	• 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±0.05mM 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±0.05mM.

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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

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.12	Minimal Reactivity	Non-sensitizer
3.11	Minimal Reactivity	Non-sensitizer
3.14	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.05	Minimal Reactivity	Non-sensitizer
3.06	Minimal Reactivity	Non-sensitizer
3.05	Minimal Reactivity	Non-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:

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

Based on HPLC-UV analysis of **AC Baby Jackharides (20963CHI)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.09% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD TG 442D: *In Vitro* Skin Sensitization

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Tradename: AC Baby Jackharides contains 2% Lactobacillus Ferment

Code: 20963CHI

CAS #: 7732-18-5 & 93333-78-9 (or) 94094-86-7 & 68333-16-4 (or) 1686112-36-6 (or) 92128-81-9

Test Request Form #: 8044

Lot #: N210504I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

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 Chemicals[1]. 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 **AC Baby Jackharides** 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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OECD TG 442D: *In Vitro* Skin Sensitization

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); Phosphate Buffered Saline (PBS)
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. Twelve test concentrations of **AC Baby Jackharides** 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 – 64 µM. 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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.

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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 EC1.5 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.

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

1. The Imax 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 EC1.5 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	32.38
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.17
AC Baby Jackharides	Non-Sensitizer	No Induction	> 1000 µM	0.31

Table 1: Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values Fg.1)

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OECD TG 442D: *In Vitro* Skin Sensitization

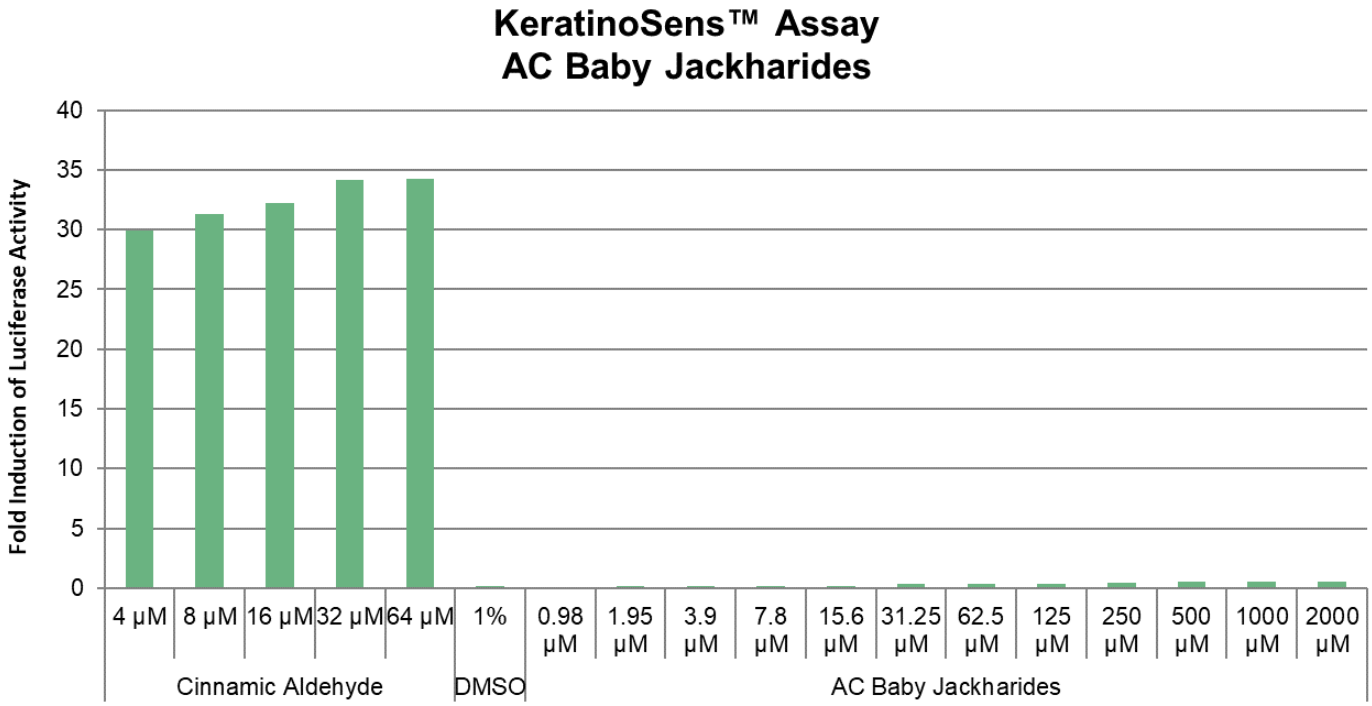

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **AC Baby Jackharides (20963CHI)** 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 **AC Baby Jackharides** can be safely used in cosmetics and personal care products at typical use levels.

Phototoxicity Assay Analysis

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Tradename: AC Baby Jackharides contains 2% Lactobacillus Ferment

Code: 20963CHI

CAS #: 7732-18-5 & 93333-78-9 (or) 94094-86-7 & 68333-16-4 (or) 1686112-36-6 (or) 92128-81-9

Test Request Form #: 8045

Lot #: N210504I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

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

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **AC Baby Jackharides** 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 0.5%, 1.5%, 5.0% and 10.0%. 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 four varying concentrations and incubated overnight at 37°C, 5% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, 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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

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Phototoxicity Assay Analysis

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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 chloropromazine, 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/cm^2 is reduced by 20% compared to the non-irradiated control tissues.

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Phototoxicity Assay Analysis

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, 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% CO_2 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% CO_2, 95% RH).

C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 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% CO_2, 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% CO_2, 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 (OD_{570}) 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%.

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Phototoxicity Assay Analysis

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 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

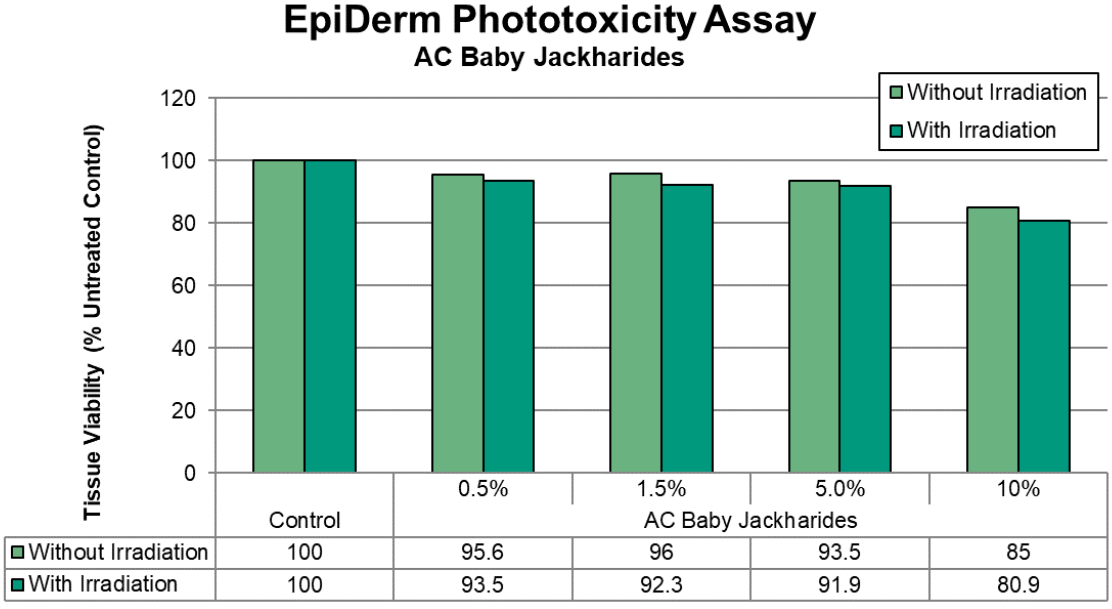
C. Test Validity

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 0.5%, 1.5%, 5.0%, and 10.0%.The negative and positive controls performed as anticipated.

There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that **AC Baby Jackharides** is not a photoirritant when used at the suggested use levels of 1.0% - 10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	95.6	96	93.5	85
With Irradiation	100	93.5	92.3	91.9	80.9

Figure 1: EpiDerm Phototoxicity Graph

Bacterial Reverse Mutation Test

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contains 2% Lactobacillus Ferment

Test Article: AC Baby Jackharides
Code Number: 20963CHI
CAS #: 7732-18-5 & 93333-78-9 (or) 94094-86-7& 68333-16-4 (or) 1686112-36-6 (or) 92128-81-9

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

Study Director: *Maureen Danaher*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 7202

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 **AC Baby Jackharides** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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Bacterial Reverse Mutation Test

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA 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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml.

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The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

VII. Conclusion

All criteria for a valid study were mete 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* WP2*uvr*A.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.

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Bacterial Reverse Mutation Test

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	31	30
	1500	32	33	33
	500	25	16	21
	150	18	18	18
	50	25	26	26
	15	28	35	32
	5.0	29	20	25
	1.5	33	39	36
Test Solution w/o S9	5000	22	22	22
	1500	21	25	23
	500	19	19	19
	150	25	23	24
	50	28	25	27
	15	38	39	39
	5.0	24	21	23
	1.5	21	22	22
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	145	123	134
	1500	166	150	158
	500	116	125	121
	150	140	127	134
	50	132	150	141
	15	125	134	130
	5.0	116	145	131
	1.5	115	126	121
Test Solution w/o S9	5000	126	146	136
	1500	100	103	102
	500	140	110	125
	150	110	116	113
	50	106	108	107
	15	116	145	131
	5.0	108	192	150
	1.5	110	115	113
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

Bacterial Reverse Mutation Test

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	Concentration µg per Plate	*TA1537*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	25	12	19
	1500	18	20	19
	500	25	16	21
	150	33	23	28
	50	44	20	32
	15	12	16	14
	5.0	10	12	11
	1.5	26	13	20
Test Solution w/o S9	5000	30	12	21
	1500	10	10	10
	500	12	10	11
	150	8	8	8
	50	16	11	14
	15	10	12	11
	5.0	20	12	16
	1.5	13	12	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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	25	11	18
	1500	13	20	17
	500	18	16	17
	150	20	21	21
	50	10	18	14
	15	24	24	24
	5.0	22	25	24
	1.5	19	19	19
Test Solution w/o S9	5000	36	45	41
	1500	25	26	26
	500	33	31	32
	150	35	32	34
	50	25	10	18
	15	22	22	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

Bacterial Reverse Mutation Test

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	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	18	17	18
	1500	26	15	21
	500	23	24	24
	150	28	21	25
	50	26	31	29
	15	21	36	29
	5.0	20	16	18
	1.5	23	19	21
Test Solution w/o S9	5000	32	42	37
	1500	22	21	22
	500	25	32	29
	150	28	25	27
	50	31	42	37
	15	13	16	15
	5.0	38	44	41
	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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Revital-Eyes
Code: 16671

Compositional Breakdown:

Ingredient	%
Water	67.00
Lactobacillus Ferment Lysate	10.00
Camellia Sinensis Leaf Extract	10.00
Punica Granatum Extract	10.00
Lactobacillus Ferment	2.00
Caffeine	1.00

Made with Lactobacillus bulgaricus and Lactobacillus acidophilus
Suggested use level: 1-10%

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Dermal and Ocular Irritation Tests

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Sample: Revital-Eyes contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment

Code: 16671

CAS #: 7732-18-5 & 68333-16-4 & 84650-60-2 & 84961-57-9 & 1686112-36-6 (or)
68333-16-4 & 58-08-2

Test Request Form/Submission #: 314

Lot #: 28324P

Sponsor: *Active Concepts, LLC; 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 **Revital-Eyes** 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% CO_2, 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.

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Dermal and Ocular Irritation Tests

II. Materials

A. Incubation Conditions: 37°C at 5% CO_2 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 H_2O

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 Concepts, 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% CO_2 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% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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Dermal and Ocular Irritation Tests

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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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) 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.

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Dermal and Ocular Irritation Tests

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

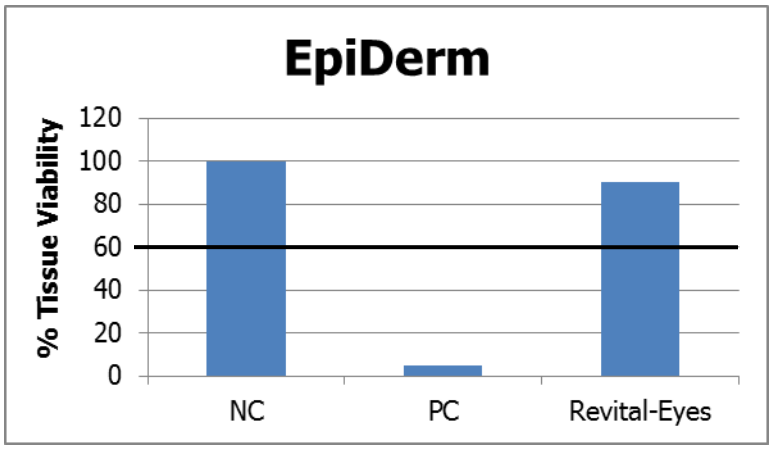

Figure 1: EpiDerm tissue viability

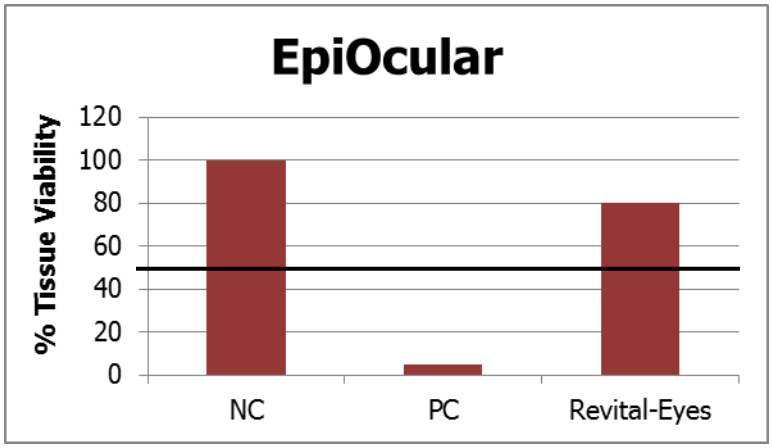

Figure 2: EpiOcular tissue viability

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: Revital-Eyes contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment

Code: 16671

CAS #: 7732-18-5 & 68333-16-4 & 84650-60-2 & 84961-57-9 & 1686112-36-6 (or)
68333-16-4 & 58-08-2
Test Request Form #: 2261

Lot #: 47147P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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 contact[1]. 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 dermatitis[2]. 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 **Revital-Eyes** 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.

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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-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-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* **Revital-Eyes** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **Revital-Eyes**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

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
• 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)	• 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±0.05mM 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±0.05mM.

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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

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.32	Minimal Reactivity	Non-sensitizer
3.26	Minimal Reactivity	Non-sensitizer
3.24	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.14	Minimal Reactivity	Non-sensitizer
3.12	Minimal Reactivity	Non-sensitizer
3.16	Minimal Reactivity	Non-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:

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

Based on HPLC-UV analysis of **Revital-Eyes (16671)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.21% 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

Tradename: Revital-Eyes contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment

Code: 16671

CAS #: 7732-18-5 & 68333-16-4 & 84650-60-2 & 84961-57-9 & 1686112-36-6 (or) 68333-16-4 & 58-08-2

Test Request Form #: 2116

Lot #: 46276P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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 Chemicals[1]. 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 **Revital-Eyes** 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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OECD TG 442D: *In Vitro* Skin Sensitization

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 **Revital-Eyes** 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 – 64 µM. 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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.

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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 EC1.5 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.

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

1. The Imax 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 EC1.5 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	31.43
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.17
Revital-Eyes	Non-Sensitizer	No Induction	> 1000 µM	0.32

Table 1: Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values Fg.1)

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OECD TG 442D: *In Vitro* Skin Sensitization

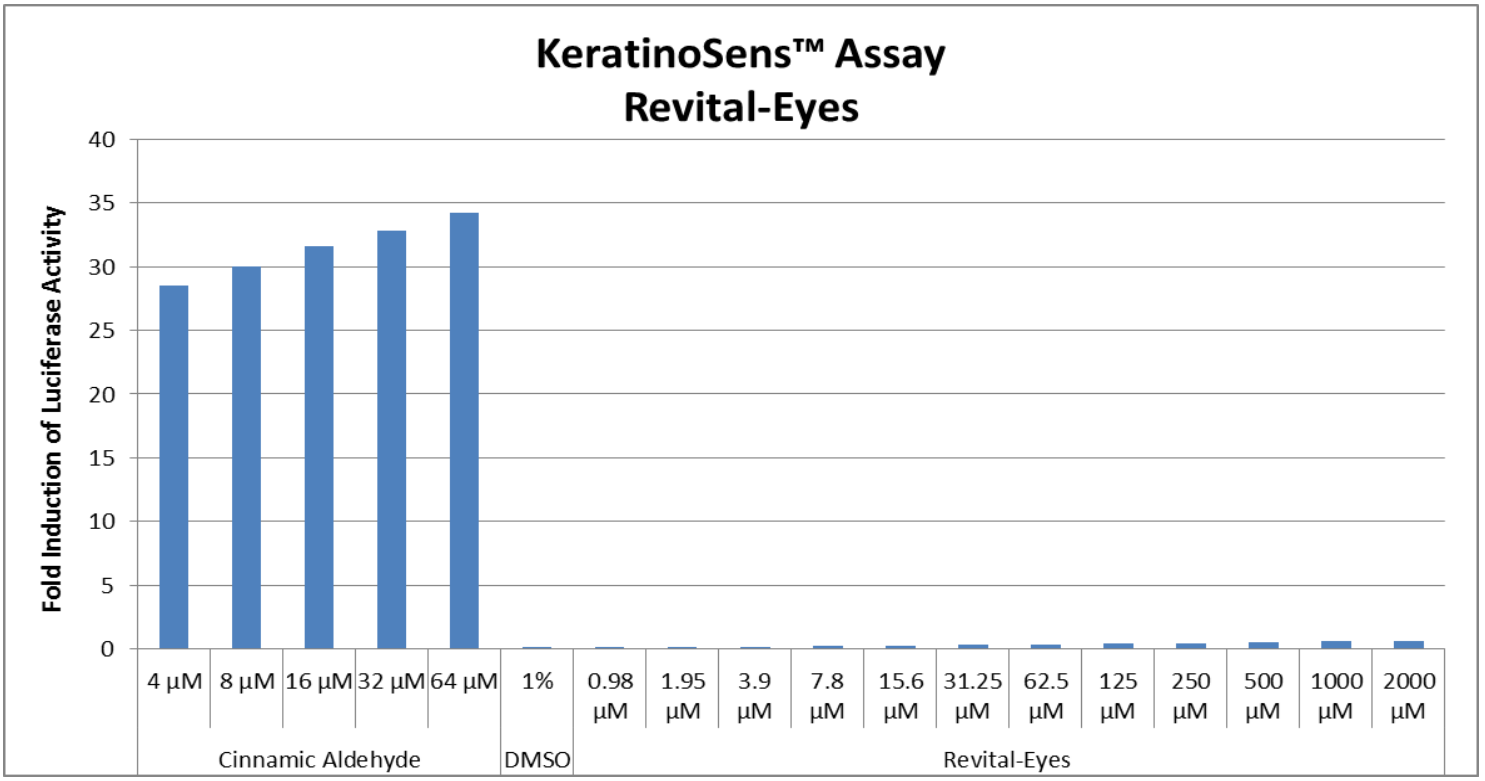

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Revital-Eyes (16671)** 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 **Revital-Eyes** can be safely used in cosmetics and personal care products at typical use levels.

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Phototoxicity Assay Analysis

Tradename: Revital-Eyes contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment

Code: 16671

CAS #: 7732-18-5 & 68333-16-4 & 84650-60-2 & 84961-57-9 & 1686112-36-6 (or) 68333-16-4 & 58-08-2

Test Request Form #: 5651

Lot #: 69782P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Michael Hovis*

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

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **Revital-Eyes** 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 0.5%, 1.5%, 5.0% and 10.0%. 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 four varying concentrations and incubated overnight at 37°C, 5% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, 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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

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Phototoxicity Assay Analysis

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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 chloropromazine, 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/cm^2 is reduced by 20% compared to the non-irradiated control tissues.

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Phototoxicity Assay Analysis

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, 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% CO_2 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% CO_2, 95% RH).

C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 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% CO_2, 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% CO_2, 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 (OD_{570}) 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%.

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Phototoxicity Assay Analysis

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 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

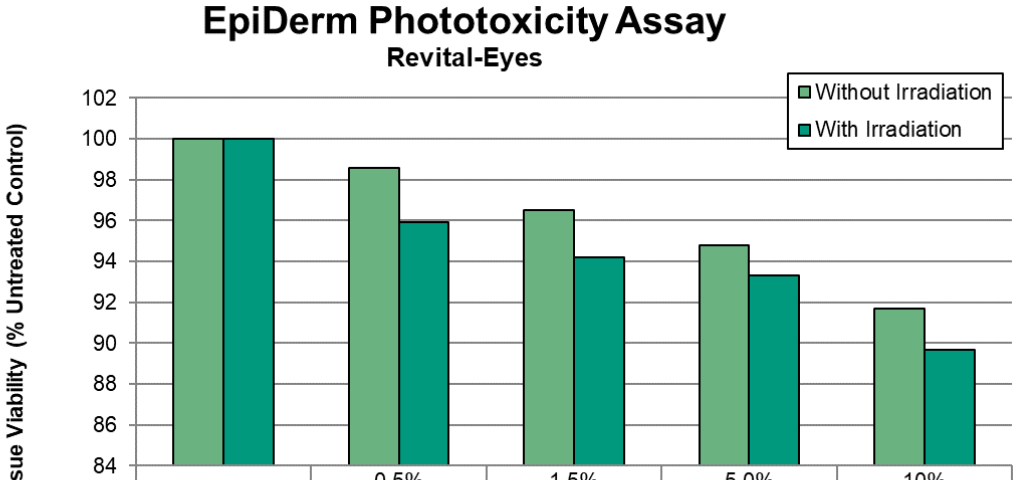
C. Test Validity

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 0.5%, 1.5%, 5.0%, and 10.0%.The negative and positive controls performed as anticipated.

There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that **Revital-Eyes** is not a photoirritant when used at the suggested use levels of 1.0% -10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	98.6	96.5	94.8	91.7
With Irradiation	100	95.9	94.2	93.3	89.7

Figure 1: EpiDerm Phototoxicity Graph

Bacterial Reverse Mutation Test

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contains 10% Lactobacillus Ferment Lysate and 2% Lactobacillus Ferment

Test Article: Revital-Eyes
Code Number: 16671
CAS #: 7732-18-5 & 68333-16-4 & 84650-60-2 & 84961-57-9 & & 1686112-36-6 (or) 68333-16-4 & 58-08-2

Sponsor:
Active Concepts, 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.Part3

Test Request Number: 644

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 **Revital-Eyes** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

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Bacterial Reverse Mutation Test

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA 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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants 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.

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B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 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 WP2*uvr*A 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 mete 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* WP2*uvr*A.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.

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Bacterial Reverse Mutation Test

Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	26	25	26
	1500	14	29	22
	500	23	16	20
	150	16	23	20
	50	20	15	18
	15	15	12	14
	5.0	12	20	16
	1.5	11	20	11
Test Solution w/o S9	5000	41	31	36
	1500	28	36	32
	500	34	17	26
	150	24	20	22
	50	25	18	22
	15	19	19	19
	5.0	24	27	25
	1.5	23	37	30
DI Water w/S9		21	10	21
DI Water w/o S9		15	21	18
2-aminoanthracen w/ S9		380	347	364
2-nitrofluorene w/o S9		178	137	158
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*TA100*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	192	332	262
	1500	236	208	222
	500	160	148	154
	150	116	204	160
	50	200	168	184
	15	144	156	150
	5.0	100	168	134
	1.5	184	148	166
Test Solution w/o S9	5000	112	112	112
	1500	134	135	134
	500	228	220	224
	150	180	172	176
	50	126	142	134
	15	136	138	137
	5.0	122	130	126
	1.5	124	148	136
DI Water w/S9		134	140	137
DI Water w/o S9		116	146	131
2-aminoanthracen w/ S9		812	813	813
Sodium azide w/o S9		1040	680	860
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*TA1537*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	16	19	18
	1500	10	18	14
	500	8	18	9
	150	6	9	8
	50	11	19	15
	15	7	26	17
	5.0	13	14	14
	1.5	18	13	16
Test Solution w/o S9	5000	42	21	32
	1500	23	32	28
	500	16	13	15
	150	7	7	7
	50	10	16	13
	15	13	7	10
	5.0	7	10	9
	1.5	13	9	11
DI Water w/S9		14	20	17
DI Water w/o S9		10	10	10
2-aminoanthracen w/ S9		50	52	51
2-aminoacridine w/o S9		58	106	82
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	25	25	25
	1500	25	39	32
	500	23	23	23
	150	13	17	15
	50	17	22	20
	15	11	14	13
	5.0	19	8	14
	1.5	10	11	11
Test Solution w/o S9	5000	27	53	40
	1500	28	36	32
	500	18	21	20
	150	29	16	23
	50	15	11	13
	15	16	23	20
	5.0	17	18	18
	1.5	16	11	14
DI Water w/S9		9	20	15
DI Water w/o S9		12	13	13
2-aminoanthracen w/ S9		86	95	91
Sodium azide w/o S9		688	960	824
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*WP2uvrA*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	16	14
	1500	20	22	21
	500	13	12	13
	150	11	11	11
	50	10	9	10
	15	12	13	13
	5.0	14	16	15
	1.5	11	13	12
Test Solution w/o S9	5000	3	1	2
	1500	4	5	5
	500	9	8	7
	150	5	5	5
	50	2	3	3
	15	2	2	2
	5.0	5	4	5
	1.5	3	3	3
DI Water w/S9		21	23	21
DI Water w/o S9		27	25	26
2-aminoanthracen w/ S9		121	130	126
Methylmethanesulfonate w/o S9		252	271	265
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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AC Barbados Gooseberry Extract G SF
Code: I11925KD

Compositional Breakdown:

Ingredient	%
Water	48.00
Glycerin	30.00
Pereskia Aculeata Callus Extract	20.00
Lactobacillus Ferment	2.00

Made with Lactobacillus acidophilus
Suggested Use Level 1-10%

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Phototoxicity Assay Analysis

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Tradename: AC Barbados Gooseberry Extract G SF contains 2% Lactobacillus Ferment

Code: I11925KD

CAS #: 7732-18-5 & 56-81-5 & 999999-99-4 & 68333-16-4 (or) 1686112-36-6

Test Request Form #: 10246

Lot #: N220203F

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **AC Barbados Gooseberry Extract G SF** would induce phototoxic irritation in the EpiDerm™ model assay.

The was performed according to the manufacturer's protocol. The test article solution was found to be a non-photoirritant at 0.5%, 1.5%, 5.0% and 10.0% concentrations. Reconstructed human epidermis were incubated in growth media for one hour to allow for tissue equilibration. The test substance was applied to the tissue inserts in four varying concentrations and incubated overnight in a humidified incubator. The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, cell viability was assessed. 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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

Introduction

A. Purpose

An i*n 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.

Phototoxicity Assay Analysis

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Materials

A. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
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; sterile Dulbecco's phosphate-buffered saline (DPBS); sterile deionized water H_2O (diH_2O)
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*) (1.0 mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1.0%)
G. Other:	Wash bottle; sterile disposable pipette tips; Parafilm; forceps

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. The EpiDerm™ tissue inserts from MatTek were in good condition, intact, and viable upon arrival.

B. Positive and Negative Controls

a. Positive Control: Concentrations of chlorpromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.
b. Negative Control: diH_2O was used as the negative control for the EpiDerm™ Photoxicity assay.

C. 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.

Method

A. Tissue Conditioning

Upon arrival, the EpiDerm™ tissue inserts were removed from their shipping medium and transferred into new culture plates with fresh media and incubated at 37°C, 5% CO_2, and 95% RH for 60 minutes. After the 60 minute incubation, the inserts were transferred into new culture plates with fresh media and dosing commenced.

B. Test Substance Exposure and Tissue Washing

a. Exposure: 50 µL of the diluted test substance in their respective concentrations were applied to 2 tissue inserts and allowed to incubate for overnight at 37°C, 5% CO_2, and 95% RH.
b. Washing: After UVA-irradiation and dark incubation, the tissue inserts were washed using DPBS and transferred to new culture plates with fresh media for an overnight incubation at 37°C, 5% CO_2, 95% RH.

C. MTT Assay

Tissue inserts were transferred into 300 µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Next, inserts were placed into sealed culture plates with 2 mL of the extraction solution and incubated in the dark for 24 hours at room temperature. Following this incubation, extraction was complete and the tissue inserts were pierced with forceps. Duplicate 200 µL aliquots of the blue formazan solution from each tissue insert was transferred into a 96-well plate for Optical Density reading at a wavelength of 570 nm (OD_{570}) with a spectrophotometer.

Phototoxicity Assay Analysis

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Acceptance Criterion

A. Positive and Negative Control
   a. Positive Control: The assay meets acceptable criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.
   b. Negative Control: The results are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 0.8.

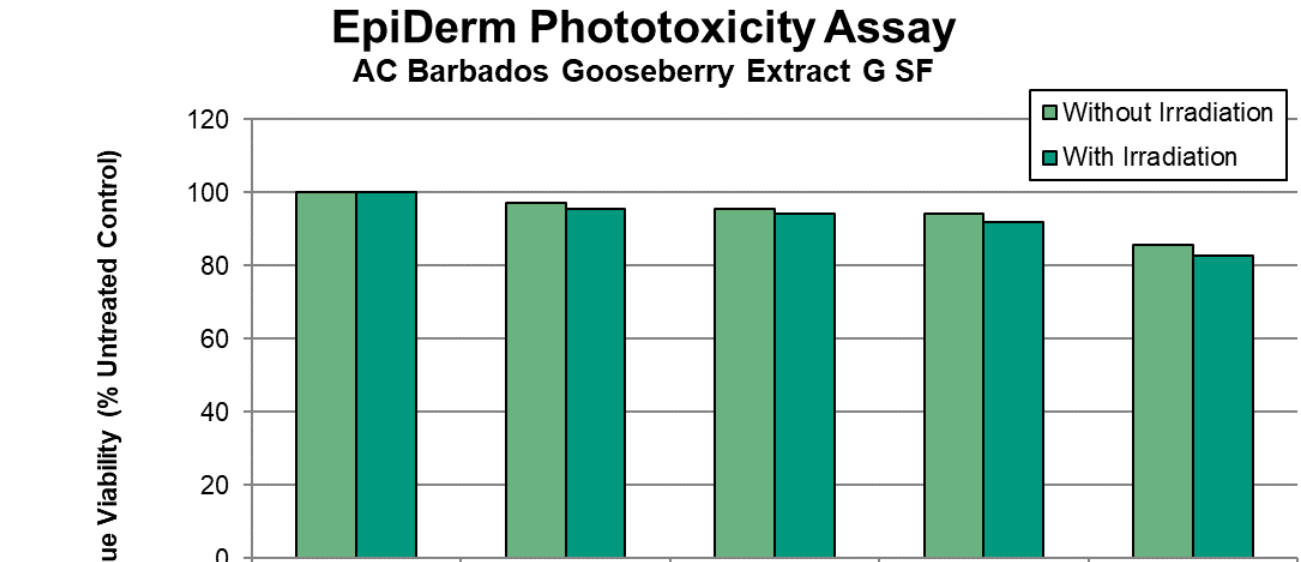
B. Standard Deviation
   Provided phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

Results

A. Test Validity and Tissue Viability Assay
   The data obtained from this study met criteria for a valid assay. The results are summarized in Figure 1. Cell viability was 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 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated. There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that AC Barbados Gooseberry Extract G SF is not a photoirritant when used at the suggested use levels of 1.0% -10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	97.2	95.6	94.1	85.8
With Irradiation	100	95.4	94.2	91.8	82.8

Figure 1. EpiDerm™ Phototoxicity Graph.

Bacterial Reverse Mutation Test

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Tradename: AC Barbados Gooseberry Extract G SF contains 2% Lactobacillus Ferment

Code: I11925KD

CAS #: 7732-18-5 & 56-81-5 & 999999-99-4 & 68333-16-4 (or) 1686112-36-6

Test Request Form #: 10252

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Monica Beltran*

Test Performed:
Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

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 AC Barbados Gooseberry Extract G SF would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^{0}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^{0}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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa = causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB = deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101 = plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A = 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.

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C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA 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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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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 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):

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

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

VII. Conclusion

All criteria for a valid study were mete 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* WP2*uvr*A.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.

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Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	14	15
	1500	25	32	29
	500	24	18	21
	150	16	17	17
	50	21	25	23
	15	12	10	11
	5.0	11	5	8
	1.5	13	25	19
Test Solution w/o S9	5000	20	32	26
	1500	17	39	28
	500	26	28	27
	150	39	24	32
	50	10	33	22
	15	21	23	22
	5.0	16	21	19
	1.5	12	24	18
DI Water w/S9		39	31	35
DI Water w/o S9		56	62	59
2-aminoanthracen w/ S9		167	153	160
2-nitrofluorene w/o S9		130	124	127
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	125	140	133
	1500	116	126	121
	500	156	143	150
	150	120	136	128
	50	159	142	151
	15	167	154	161
	5.0	143	161	152
	1.5	128	130	129
Test Solution w/o S9	5000	126	146	136
	1500	165	140	153
	500	133	125	129
	150	147	124	136
	50	188	196	192
	15	152	141	147
	5.0	119	104	112
	1.5	107	127	117
DI Water w/S9		116	101	109
DI Water w/o S9		86	98	92
2-aminoanthracen w/ S9		602	644	623
Sodium azide w/o S9		583	641	612
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	30	25	28
	1500	16	12	14
	500	14	17	16
	150	13	13	13
	50	19	21	20
	15	10	12	11
	5.0	19	23	21
	1.5	14	17	16
Test Solution w/o S9	5000	19	25	22
	1500	26	32	29
	500	19	25	23
	150	26	17	22
	50	14	18	16
	15	25	29	27
	5.0	27	26	27
	1.5	20	18	19
DI Water w/S9		14	17	16
DI Water w/o S9		26	28	27
2-aminoanthracen w/ S9		390	362	376
2-aminoacridine w/o S9		131	126	129
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	33	13	23
	1500	11	9	10
	500	13	14	14
	150	20	25	23
	50	16	14	15
	15	8	20	14
	5.0	19	20	20
	1.5	17	12	15
Test Solution w/o S9	5000	15	16	16
	1500	11	19	15
	500	6	15	11
	150	24	18	21
	50	30	24	27
	15	23	22	23
	5.0	19	22	21
	1.5	25	20	23
DI Water w/S9		38	28	33
DI Water w/o S9		12	16	14
2-aminoanthracen w/ S9		145	157	151
Sodium azide w/o S9		715	708	712
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	*WP2uvrA*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	14	25	20
	1500	12	13	13
	500	14	15	15
	150	10	22	16
	50	11	25	18
	15	17	12	15
	5.0	16	10	13
	1.5	11	13	12
Test Solution w/o S9	5000	20	13	17
	1500	10	13	12
	500	8	16	12
	150	20	17	19
	50	26	23	25
	15	19	20	20
	5.0	24	34	29
	1.5	39	22	31
DI Water w/S9		28	17	23
DI Water w/o S9		13	8	11
2-aminoanthracen w/ S9		152	181	167
Methylmethanesulfonate w/o S9		242	257	250
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

OECD 442C: *In Chemico* Skin Sensitization

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Tradename: AC Barbados Gooseberry Extract G SF contains 2% Lactobacillus Ferment

Code: I11925KD

CAS #: 7732-18-5 & 56-81-5 & 999999-99-4 & 68333-16-4 (or) 1686112-36-6

Test Request Form #: 10243

Lot #: N220203F

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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 contact[1]. 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 dermatitis[2]. 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 **AC Barbados Gooseberry Extract G 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.

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Materials

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

Methods

A. Solution Preparation

a. 0.667 mM Cysteine Peptide in 100 mM Phosphate Buffer (pH 7.5)
b. 0.667 mM Lysine Peptide in 100 mM Ammonium Acetate Buffer (pH 10.2)
c. 100 mM Cinnamic Aldehyde in Acetonitrile
d. 100 mM* **AC Barbados Gooseberry Extract G SF** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **AC Barbados Gooseberry Extract G SF**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

B. Reference Controls

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

C. Sample, Reference Control, and Co-Elution Control Preparation

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

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM Test Chemical

• 750 µL Cysteine Peptide Solution
  ○ *Or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls*
• 200 µL Acetonitrile
• 50 µL Test Chemical Solution
  ○ *Or Acetonitrile for Reference Controls*
	• 750 µL Lysine Peptide Solution
	  ○ *Or 100 mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls*
	• 250 µL Test Chemical Solution
	  ○ *Or Acetonitrile for Reference Controls*

D. Calibration Curve

a. Standards are prepared in a solution of 20% Acetonitrile:Buffer
   i. For the Cysteine peptide using the phosphate buffer, pH 7.5
   ii. 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

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E. HPLC Analysis
   a. 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
   b. Absorbance is measured at 220 nm
   c. Flow Conditions:

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

F. Calculations
   a. Percent peptide depletion is determined by the following equation:

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

Data and Reporting

A. Acceptance Criteria
   a. The following criteria must be met for a run to be considered valid:
      i. Standard calibration curve should have an $r^2 > 0.99$.
      ii. 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.
      iii. 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%.
   b. The following criteria must be met for a test chemical's results to be considered valid:
      i. Maximum standard deviation should be <1 4.9 for percent cysteine depletion and < 11.6 for percent lysine depletion.
      ii. Mean peptide concentration of the three reference control C should be 0.50 ± 0.05 mM.

B. Prediction Model
   a. Cysteine 1:10/Lysine 1:50 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

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b. 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

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. The measured values of percent depletion, reactivity class, and prediction for each assay are outlined in Table 1 and Table 2.

Table 1. Cysteine 1:10/Lysine 1:50 Prediction Model.

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
3.13	Minimal Reactivity	Non-sensitizer
3.12	Minimal Reactivity	Non-sensitizer
3.09	Minimal Reactivity	Non-sensitizer

Table 2. Cysteine 1:10 Prediction Model.

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
3.02	Minimal Reactivity	Non-sensitizer
3.01	Minimal Reactivity	Non-sensitizer
3.05	Minimal Reactivity	Non-sensitizer

Discussion

Based on HPLC-UV analysis of **AC Barbados Gooseberry Extract G SF (I11925KD)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.07% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD 442D: *In Vitro* Skin Sensitization

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CAS #: 7732-18-5 & 56-81-5 & 999999-99-4 & 68333-16-4 (or) 1686112-36-6

Test Request Form #: 10245

Lot #: N220203F

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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 Chemicals[1]. 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 **AC Barbados Gooseberry Extract G 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, 5% CO_2, and 95% relative humidity (RH)
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Phosphate Buffered Saline (PBS)
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. Twelve test concentrations of **AC Barbados Gooseberry Extract G 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 – 64 µM. The negative control was a 1.0% test concentration of DMSO.

24 hours 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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

A. Acceptance Criteria

a. 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).

b. 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.

c. 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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B. A KeratinoSens™ prediction is considered positive if the following conditions are met:
   a. The I_{max} is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
   b. 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)
   c. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
   d. There is an apparent overall dose-response for luciferase induction

Results

Table 1. Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values in Figure 1).

Compound	*Classification*	*$EC_{1.5}$ (µM)*	*IC_{50}*	*I_{max}*
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.64
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.19
AC Barbados Gooseberry Extract G SF	Non-Sensitizer	No Induction	> 1000 µM	0.42

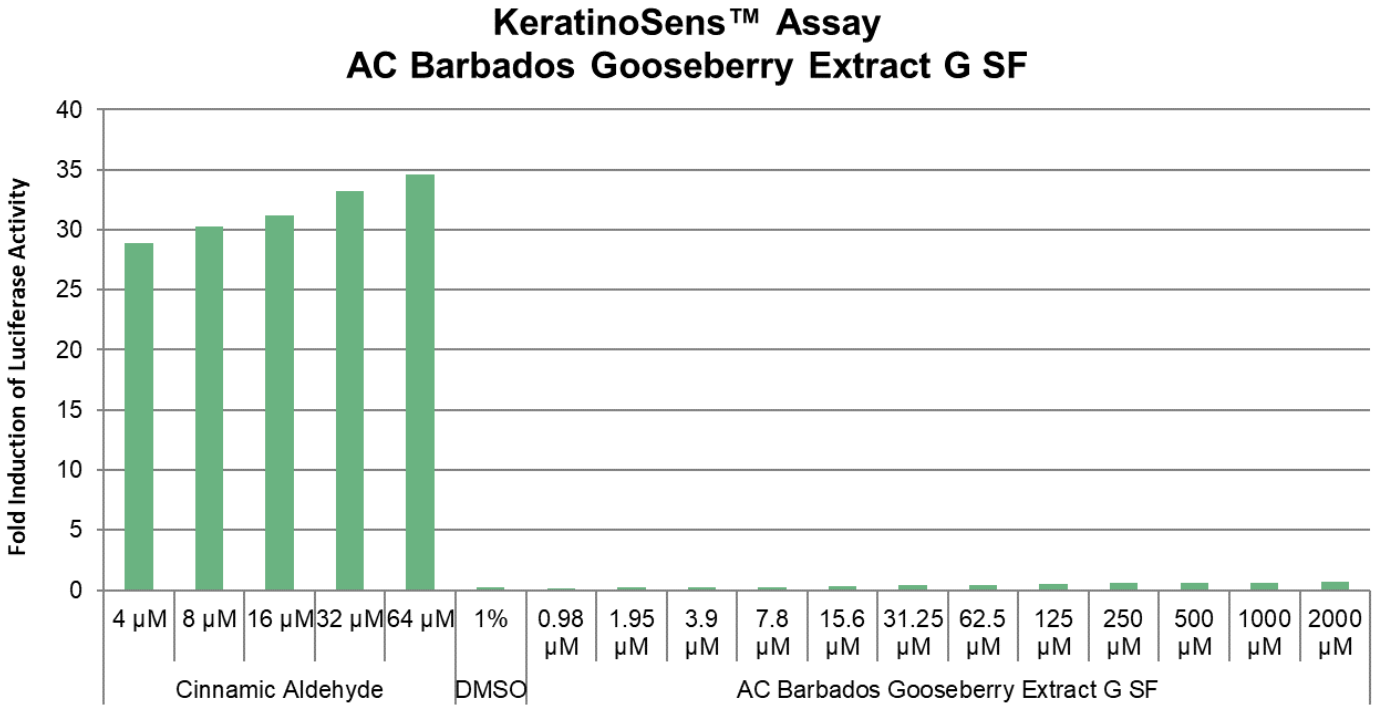

Figure 1. Effect of Test Conditions on Luciferase Fold Induction.

Discussion

As shown in the results, **AC Barbados Gooseberry Extract G SF (I11925KD)** 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 **AC Barbados Gooseberry Extract G SF** can be safely used in cosmetics and personal care products at typical use levels.

Compositional Breakdown

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AC AlfalfaBoost
Code: 20988

Compositional Breakdown:

Ingredient	%
Medicago Sativa (Alfalfa) Callus Culture Conditioned Media Extract	98.00
Lactobacillus Ferment Lysate	2.00

made with Lactobacillus acidophilius
suggested us level: 1-10%

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential 26 fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of EU Cosmetic Regulation(EC) No. 1223/2009	
INCI NAME	CAS NUMBER
Alpha-Isomethyl Ionone	127-51-5
Amyl Cinnamal	122-40-7
Amylcinnamyl Alcohol	101-85-9
Anise Alcohol	105-13-5
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Butylphenyl Methylpropional	80-54-6
Cinnamal	104-55-2
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9
Coumarin	91-64-5
Eugenol	97-53-0
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geraniol	106-24-1
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Hydroxyisohexyl 3-Cyclohexene Carboxaldehyde (Lyral)	31906-04-4
Isoeugenol	97-54-1
Limonene (sum of d, l and dl)	5989-27-5
Linalool	78-70-6
Methyl 2-Octynoate	111-12-6

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Dermal and Ocular Irritation Tests

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 9931

Lot #: N22114D

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

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 **AC AlfalfaBoost** 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 **non-irritating**. 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% CO_2, 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-irritant**. The negative and positive controls performed as anticipated.

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Dermal and Ocular Irritation Tests

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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.

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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 Concepts, 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% CO_2 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% CO_2 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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

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Dermal and Ocular Irritation Tests

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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 Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

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Dermal and Ocular Irritation Tests

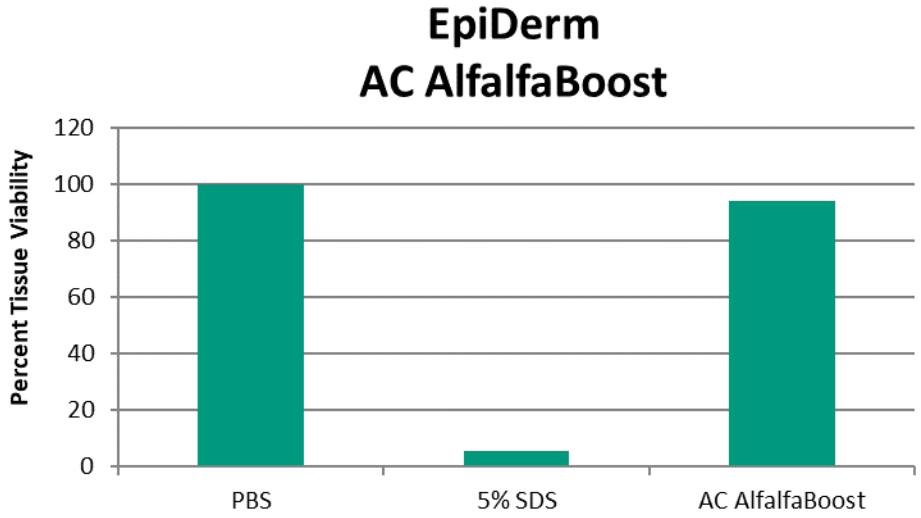

Figure 1: EpiDerm tissue viability

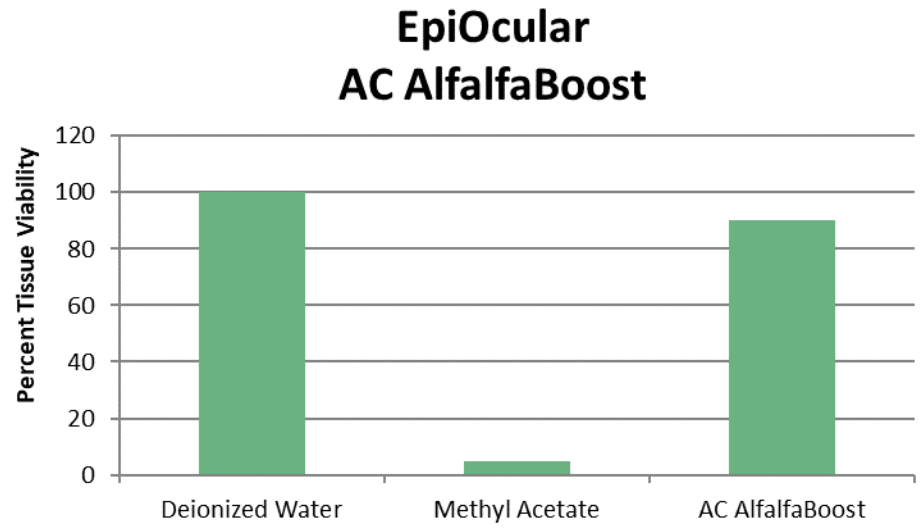

Figure 2: EpiOcular tissue viability

Bacterial Reverse Mutation Test

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 10199

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Monica Beltran*

Test Performed:
Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

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 AC AlfalfaBoost would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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.

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C. Preparation of Tester strains

Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA 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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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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 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):

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

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

VII. Conclusion

All criteria for a valid study were mete 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* WP2*uvr*A.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.

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Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	20	18
	1500	12	13	13
	500	18	20	19
	150	22	21	22
	50	14	19	17
	15	20	25	23
	5.0	15	21	18
	1.5	21	23	22
Test Solution w/o S9	5000	17	20	19
	1500	21	16	19
	500	17	35	26
	150	20	39	30
	50	22	27	25
	15	15	12	14
	5.0	16	18	17
	1.5	29	26	28
DI Water w/S9		22	17	20
DI Water w/o S9		62	66	64
2-aminoanthracen w/ S9		163	152	158
2-nitrofluorene w/o S9		122	119	121
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	135	144	140
	1500	112	136	124
	500	165	156	161
	150	147	158	153
	50	140	120	130
	15	156	160	158
	5.0	139	147	143
	1.5	110	117	114
Test Solution w/o S9	5000	126	142	134
	1500	147	135	141
	500	146	133	140
	150	130	156	143
	50	129	146	138
	15	122	130	126
	5.0	117	110	114
	1.5	112	120	116
DI Water w/S9		95	102	99
DI Water w/o S9		83	100	92
2-aminoanthracen w/ S9		632	620	626
Sodium azide w/o S9		558	616	587
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	10	12	11
	1500	14	18	16
	500	16	12	14
	150	11	9	10
	50	14	8	12
	15	12	15	14
	5.0	11	10	11
	1.5	13	14	14
Test Solution w/o S9	5000	13	25	18
	1500	14	14	14
	500	13	15	14
	150	11	19	15
	50	12	10	11
	15	14	20	17
	5.0	18	17	18
	1.5	10	11	11
DI Water w/S9		15	8	12
DI Water w/o S9		20	27	24
2-aminoanthracen w/ S9		362	377	370
2-aminoacridine w/o S9		120	119	120
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	10	12	11
	1500	17	10	14
	500	13	14	14
	150	15	14	15
	50	17	12	15
	15	15	17	16
	5.0	11	15	13
	1.5	10	15	13
Test Solution w/o S9	5000	18	16	17
	1500	10	23	17
	500	13	14	14
	150	10	6	8
	50	12	10	11
	15	18	12	15
	5.0	18	7	13
	1.5	13	20	17
DI Water w/S9		33	35	34
DI Water w/o S9		8	17	13
2-aminoanthracen w/ S9		152	163	158
Sodium azide w/o S9		737	724	731
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	*WP2uvrA*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	16	18	17
	1500	11	15	13
	500	13	12	13
	150	12	11	12
	50	14	15	15
	15	10	16	13
	5.0	11	10	11
	1.5	17	13	15
Test Solution w/o S9	5000	18	17	18
	1500	14	12	13
	500	10	14	12
	150	16	18	17
	50	12	11	12
	15	10	11	11
	5.0	21	18	20
	1.5	14	28	21
DI Water w/S9		27	20	24
DI Water w/o S9		14	10	12
2-aminoanthracen w/ S9		152	169	161
Methylmethanesulfonate w/o S9		220	244	232
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

Phototoxicity Assay Analysis

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 10186

Lot #: N230119B

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **AC AlfalfaBoost** would induce phototoxic irritation in the EpiDerm™ model assay.

The was performed according to the manufacturer's protocol. The test article solution was found to be a non-photoirritant at 0.5%, 1.5%, 5.0% and 10.0% concentrations. Reconstructed human epidermis were incubated in growth media for one hour to allow for tissue equilibration. The test substance was applied to the tissue inserts in four varying concentrations and incubated overnight in a humidified incubator. The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, cell viability was assessed. 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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

Introduction

A. Purpose

An i*n 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.

Phototoxicity Assay Analysis

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Materials

A. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
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; sterile Dulbecco's phosphate-buffered saline (DPBS); sterile deionized water H_2O (diH_2O)
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*) (1.0 mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1.0%)
G. Other:	Wash bottle; sterile disposable pipette tips; Parafilm; forceps

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. The EpiDerm™ tissue inserts from MatTek were in good condition, intact, and viable upon arrival.

B. Positive and Negative Controls

a. Positive Control: Concentrations of chlorpromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.
b. Negative Control: diH_2O was used as the negative control for the EpiDerm™ Photoxicity assay.

C. 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.

Method

A. Tissue Conditioning

Upon arrival, the EpiDerm™ tissue inserts were removed from their shipping medium and transferred into new culture plates with fresh media and incubated at 37°C, 5% CO_2, and 95% RH for 60 minutes. After the 60 minute incubation, the inserts were transferred into new culture plates with fresh media and dosing commenced.

B. Test Substance Exposure and Tissue Washing

a. Exposure: 50 µL of the diluted test substance in their respective concentrations were applied to 2 tissue inserts and allowed to incubate for overnight at 37°C, 5% CO_2, and 95% RH.
b. Washing: After UVA-irradiation and dark incubation, the tissue inserts were washed using DPBS and transferred to new culture plates with fresh media for an overnight incubation at 37°C, 5% CO_2, 95% RH.

C. MTT Assay

Tissue inserts were transferred into 300 µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Next, inserts were placed into sealed culture plates with 2 mL of the extraction solution and incubated in the dark for 24 hours at room temperature. Following this incubation, extraction was complete and the tissue inserts were pierced with forceps. Duplicate 200 µL aliquots of the blue formazan solution from each tissue insert was transferred into a 96-well plate for Optical Density reading at a wavelength of 570 nm (OD_{570}) with a spectrophotometer.

Phototoxicity Assay Analysis

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Acceptance Criterion

A. Positive and Negative Control
   a. Positive Control: The assay meets acceptable criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.
   b. Negative Control: The results are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 0.8.

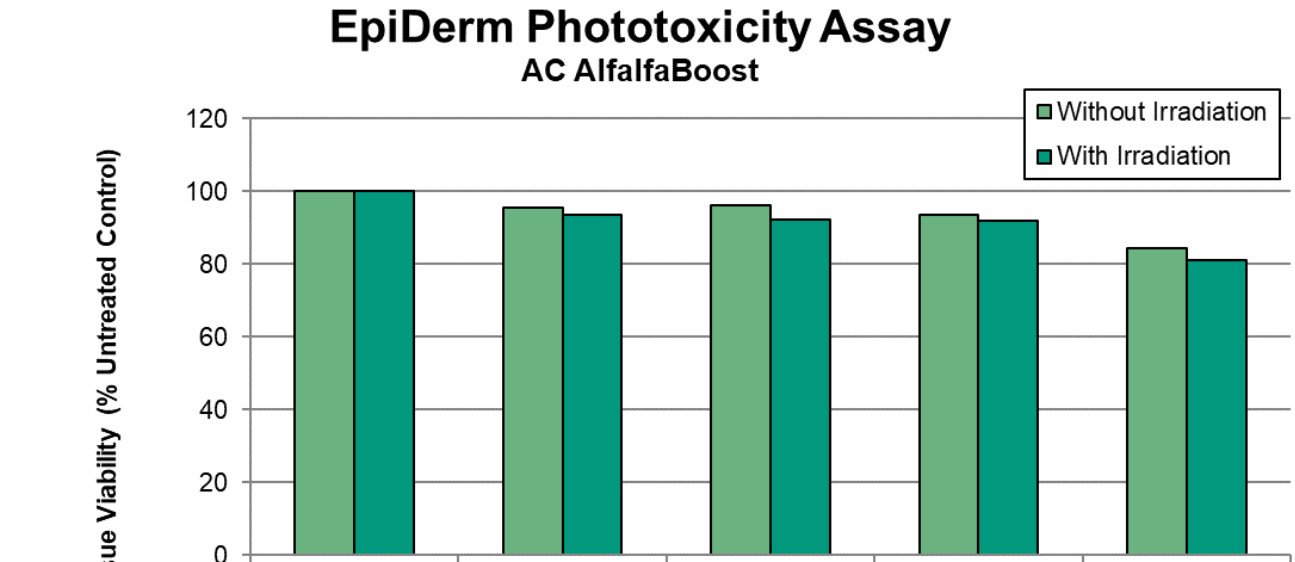
B. Standard Deviation
   Provided phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

Results

A. Test Validity and Tissue Viability Assay
   The data obtained from this study met criteria for a valid assay. The results are summarized in Figure 1. Cell viability was 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 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

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 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated. There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that AC AlfalfaBoost is not a photoirritant when used at the suggested use levels of 1.0% - 10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	95.7	96.1	93.6	84.3
With Irradiation	100	93.6	92.4	92	81

Figure 1. EpiDerm™ Phototoxicity Graph.

OECD 442D: *In Vitro* Skin Sensitization

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 10185

Lot #: N230119B

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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 Chemicals[1]. 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 **AC AlfalfaBoost** 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, 5% CO_2, and 95% relative humidity (RH)
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Phosphate Buffered Saline (PBS)
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. Twelve test concentrations of **AC AlfalfaBoost** 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 – 64 µM. The negative control was a 1.0% test concentration of DMSO.

24 hours 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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

A. Acceptance Criteria

a. 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).

b. 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.

c. 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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B. A KeratinoSens™ prediction is considered positive if the following conditions are met:
   a. The I_{max} is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
   b. 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)
   c. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
   d. There is an apparent overall dose-response for luciferase induction

Results

Table 1. Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values in Figure 1).

Compound	*Classification*	*$EC_{1.5}$ (µM)*	*IC_{50}*	*I_{max}*
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.18
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.15
AC AlfalfaBoost	Non-Sensitizer	No Induction	> 1000 µM	0.35

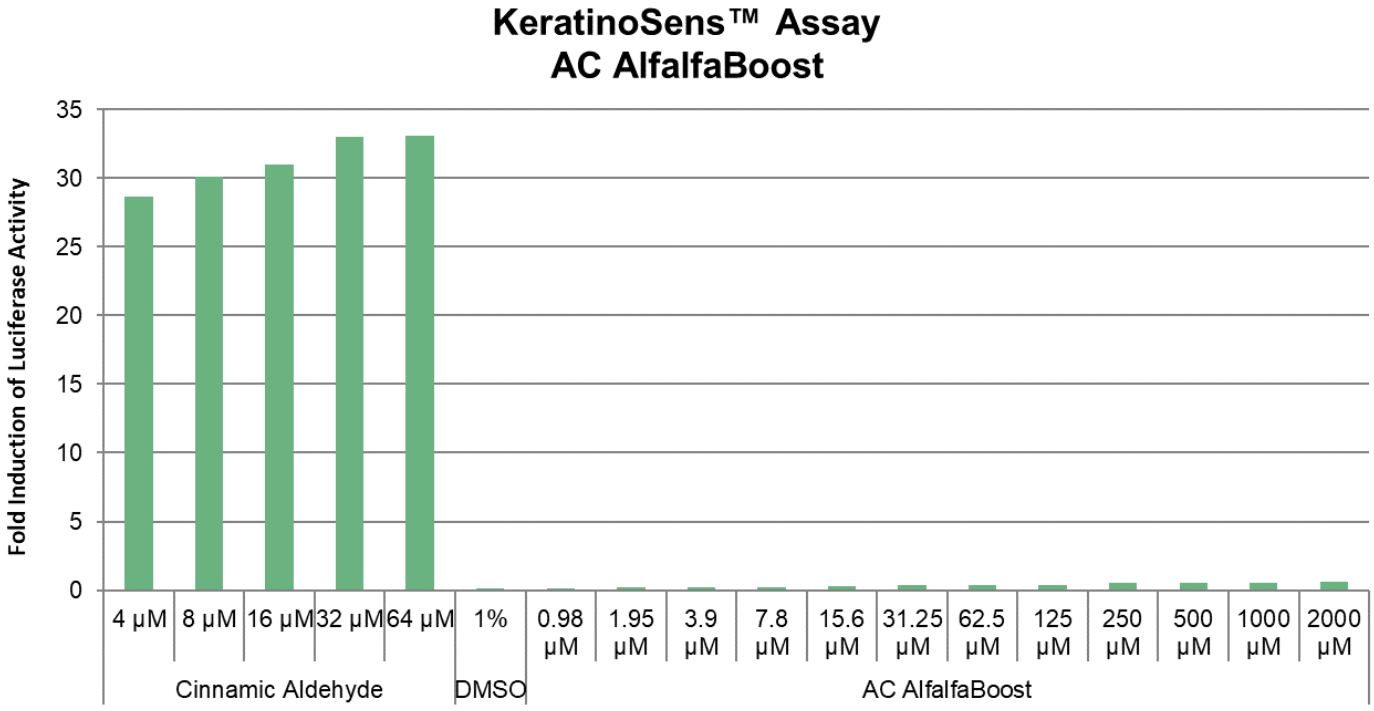

Figure 1. Effect of Test Conditions on Luciferase Fold Induction.

Discussion

As shown in the results, **AC AlfalfaBoost (20988)** 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 **AC AlfalfaBoost** can be safely used in cosmetics and personal care products at typical use levels.

OECD 442C: *In Chemico* Skin Sensitization

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 10181

Lot #: N230119B

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Daniel Shill*

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 contact[1]. 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 dermatitis[2]. 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 **AC AlfalfaBoost** 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.

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Materials

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

Methods

A. Solution Preparation

a. 0.667 mM Cysteine Peptide in 100 mM Phosphate Buffer (pH 7.5)
b. 0.667 mM Lysine Peptide in 100 mM Ammonium Acetate Buffer (pH 10.2)
c. 100 mM Cinnamic Aldehyde in Acetonitrile
d. 100 mM* **AC AlfalfaBoost** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **AC AlfalfaBoost**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

B. Reference Controls

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

C. Sample, Reference Control, and Co-Elution Control Preparation

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

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM Test Chemical

• 750 µL Cysteine Peptide Solution
  ○ *Or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls*
• 200 µL Acetonitrile
• 50 µL Test Chemical Solution
  ○ *Or Acetonitrile for Reference Controls*
	• 750 µL Lysine Peptide Solution
	  ○ *Or 100 mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls*
	• 250 µL Test Chemical Solution
	  ○ *Or Acetonitrile for Reference Controls*

D. Calibration Curve

a. Standards are prepared in a solution of 20% Acetonitrile:Buffer
   i. For the Cysteine peptide using the phosphate buffer, pH 7.5
   ii. 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

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E. HPLC Analysis
   a. 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
   b. Absorbance is measured at 220 nm
   c. Flow Conditions:

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

F. Calculations
   a. Percent peptide depletion is determined by the following equation:

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

Data and Reporting

A. Acceptance Criteria
   a. The following criteria must be met for a run to be considered valid:
      i. Standard calibration curve should have an $r^2 > 0.99$.
      ii. 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.
      iii. 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%.
   b. The following criteria must be met for a test chemical's results to be considered valid:
      i. Maximum standard deviation should be <1 4.9 for percent cysteine depletion and < 11.6 for percent lysine depletion.
      ii. Mean peptide concentration of the three reference control C should be 0.50 ± 0.05 mM.

B. Prediction Model
   a. Cysteine 1:10/Lysine 1:50 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

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b. 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

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. The measured values of percent depletion, reactivity class, and prediction for each assay are outlined in Table 1 and Table 2.

Table 1. Cysteine 1:10/Lysine 1:50 Prediction Model.

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
3.14	Minimal Reactivity	Non-sensitizer
3.09	Minimal Reactivity	Non-sensitizer
3.12	Minimal Reactivity	Non-sensitizer

Table 2. Cysteine 1:10 Prediction Model.

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
2.99	Minimal Reactivity	Non-sensitizer
3.02	Minimal Reactivity	Non-sensitizer
3.02	Minimal Reactivity	Non-sensitizer

Discussion

Based on HPLC-UV analysis of **AC AlfalfaBoost (20988)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.06% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD 456: H295R Steroidogenesis Assay

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Tradename: AC AlfalfaBoost contains 2% Lactobacillus Ferment Lysate

Code: 20988

CAS #: 84082-36-0 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 11513

Lot #: N230821G

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
OECD 456: H295R Steroidogenesis Assay

Introduction

Endocrine disrupting agents are substances that interfere with the endocrine system and are associated with several negative health effects. Located throughout the body, endocrine glands release hormones into circulation and these signaling molecules are vital to maintaining homeostasis. Endocrine glands and hormones are extremely sensitive and small disruptions to the structure or function of any aspect of the endocrine system significantly alter physiological processes. Endocrine disruptors work through one or a combination of the following mechanisms: 1) mimicking, or partly mimicking, naturally occurring hormones, potentially leading to over production, 2) binding to a receptor and blocking a naturally occurring hormone from binding, and/or 3) interfering with or preventing the synthesis or utilization of natural hormones and/or receptors. The OECD 456: H295R Steroidogenesis Assay was developed as an *in vitro* test to screen for test article effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T).

A H295R Steroidogenesis Assay was conducted to determine the potential of **AC AlfalfaBoost** to induce or inhibit the production of 17β-estradiol and testosterone.

Assay Principle

The *in vitro* H295R Steroidogenesis Assay utilizes a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 "*in vitro* assay, providing mechanistic data" to screen for test article effects on steroidogenesis, but does not aim to provide specific mechanistic information concerning the interaction of the test substance with the endocrine system. Moreover, the H295R assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic pituitary-gonadal axis. The objective of the H295R Steroidogenesis Assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone (T). The H295R assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions from cholesterol to the production of E2 and/or T. Human adreno-carcinoma H295R cells are utilized because the cell line expresses genes that encode all key steroidogenesis enzymes, enables the detection of both increases and decreases in the production of both E2 and T, and permits the direct assessment of the potential impact of a test article on cell viability/cytotoxicity. The purpose of the assay is the detection of substances that affect E2 and T production by distinguishing test articles that typically inhibit or induce key enzymes of the steroidogenesis pathway.

OECD 456: H295R Steroidogenesis Assay

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The human adreno-carcinoma H295R cells are exposed to different concentrations of the test substance. After the incubation period, the medium is removed from each well and cellular viability is immediately analyzed. Next, concentrations of E2 and T in the medium are measured.

Materials

A. Kit:	Estradiol ELISA Kit (Cayman Chemical; 501890)*; DetectX® Testosterone ELISA Kit (Arbor Assays; K032-H)*; CyQUANT™ MTT Cell Viability Assay (ThermoFisher; V13154)*
B. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
C. Equipment:	Forma Humidified Incubator; ESCO Biosafety Laminar Flow Hood; Synergy HT Microplate Reader; Pipettes; Light Microscope; Weigh Boats; Chemical Spatula; Analytical Balance
D. Cell Line:	Human Adreno-Carcinoma Cell (NCI-H295R [H295R]) (ATCC; CRL-2128)*
E. Media/Buffers:	DMEM: F-12 Medium (ATCC; 30-2006)*; ITS+ Premix Universal Culture Supplement (Corning®; 354352)*; Nu-Serum™ Growth Medium Supplement (Corning®; 355100)*; Dimethyl sulfoxide (DMSO); Deionized water
F. Reagents:	Forskolin (ThermoFisher; J63292.MF)*; Prochloraz (ThermoFisher; P21371G)* Phosphate Buffered Saline (PBS); Trypsin; Trypsin-EDTA; Methanol; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT Assay; ThermoFisher; V13154); SDS-HCl Solution (sodium dodecyl sulfate hydrochloride) (MTT Assay; ThermoFisher; V13154)
G. Culture Plate:	24 Well Tissue Culture Treated Microplates; Tissue Culture Treated Flasks
H. Software:	Excel Analysis ToolPak (Microsoft)
I. Other:	Sterile disposable pipette tips

**Or suitable alternatives, subject to change without notice based off vendor availability*

Methods

One source vial of H295R cells were initiated from cryopreservation and expanded three passages to establish a lot for screening and cells were expanded five times before used for testing or frozen for storage. Cells cultured from the expanded stock did not exceed 10 passages. Cells were passaged and maintained in Complete Media (DMEM: F-12 Medium + 1.0% ITS+ Premix Universal Culture Supplement + 2.5% Nu-Serum™ Growth Medium Supplement). The maintenance, expansion, and cryopreservation standard operating procedures were adopted directly from the guideline specifications. A quality control test plate to establish acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement assays, and variability between replicate hormone measures of the same sample were performed and met criteria according to the guideline specifications.

Cells utilized for testing were seeded at a target density of 200,000 to 300,000 cells per mL of Complete Media resulting in approximately 50-60% confluency in the wells at 24 hours to achieve the optimal cell density for hormone production. After an acclimation period of 24 hours, cells were exposed for 48 hours to seven concentrations of **AC AlfalfaBoost**, diluted with DMSO, in triplicate. Cells were also exposed to Complete Media and positive, negative, and solvent controls. Prepared in DMSO, Forskolin (10 µM) and Prochloraz (1.0 µM) were utilized as positive and negative controls as they are known inducers and inhibitors, respectively, of E2 and T synthesis. DMSO was utilized as the solvent control. At the end of the 48-hour exposure period, the media is removed from each well and stored in aliquots at -80°C until hormone analysis. Cell viability in each well is analyzed immediately after removal of media. Conclusions regarding the ability of a test article to affect steroidogenesis are based on at least two independent experimental runs.

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To determine cell viability, fresh Complete Media with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to all wells, except for the positive control wells which received 70% Methanol in Complete Media provided Methanol is a known cytotoxicity inducer. After a 4-hour incubation period at 37°C, the SDS-HCl solution was added to all wells and the plate was incubated again at 37°C for 4 hours after which the absorbance was read at 570 nm. Cell viability data is displayed as Mean Absorbance Units (MAU) and reflects averages of the triplicates for independent experimental runs. The results were analyzed utilizing a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Conditions demonstrating viability lower than 80% were not included in the final data analysis. The percentage of viable cells was calculated with the following equation:

$$Viable\ Cells\ (\%) = \frac{MAU_{Sample} - MAU_{Methanol}}{MAU_{DMSO} - MAU_{Methanol}} \times 100$$

After cellular viability was determined, 17β-estradiol (E2) and testosterone (T) concentration levels in the media were determined with an Estradiol ELISA Kit (Limit of Quantification 6.0 pg/mL) and DetectX® Testosterone ELISA Kit (Limit of Quantification 30.6 pg/mL), respectively. Frozen media aliquots were thawed at room temperature and both ELISAs were performed according to the manufacturers' protocols. Briefly, thawed media aliquots were loaded onto the plate along with prepared standards. Next, conjugates and antibodies were added to appropriate wells and allowed to incubate at room temperature. After the incubation, wells were washed, and antibody detection reagents were added to each well followed by stop solutions to stop the antibody detection reactions. Lastly, absorbance values were measured.

The criteria for a negative, positive, or equivocal run are provided in Diagram 1. A minimum of two independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at $\log_{10}$ intervals with 10^{-3} M being the maximum concentration. If the test article is soluble, and not cytotoxic at any of the tested concentrations, and the first run was negative for all concentrations, then it is to be confirmed in one more run using the same conditions as the first run was conducted (Table 1). If the results of the first run are equivocal or positive, the test should be repeated as indicated in Table 1 and footnote 'a' to Diagram 1, by refining the selected test concentrations. Test concentrations in runs two and three (if applicable) should be adjusted on the basis of the results of the initial run bracketing concentrations that elicited an effect using ½-log concentration spacing (e.g. if the original run of 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM resulted in inductions at 1 and 10 µM, the concentrations tested in the second run should be 0.1, 0.3, 1, 3, 10, 30, 100 µM), unless lower concentrations need to be employed to achieve a Lowest-Observed-Effect-Concentration (LOEC). In the latter case, at least five concentrations below the lowest concentration tested in the first run should be used in the second run using a ½-log scale. If the assay is equivocal or positive, the LOEC is reported. If the assay is negative, the highest concentration tested is reported as the No-Observed-Effect-Concentration (NOEC).

If the second run does not confirm the first run, a third experiment is to be conducted using the original testing conditions. Equivocal results in the first run are considered negative if the observed effect could not be confirmed in any of the two subsequent runs. Equivocal results are considered as weak positive responses (effect) when the response can be confirmed in at least two more runs within a ± 1 concentration increment (Diagram 1; Table 1). Non monotonic concentration response patterns are rare but possible. If reproducible effects are seen at lower concentrations which then dissipate at greater concentrations and/or return at the top concentrations, this data should not be disregarded and should be included in the study report.

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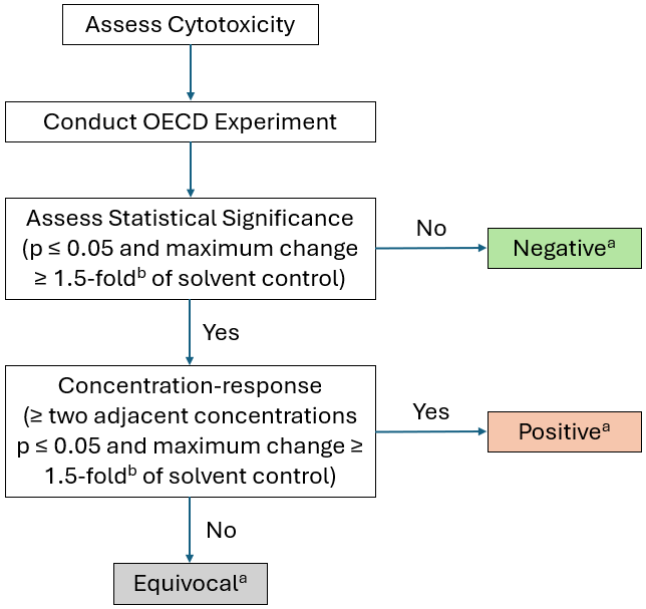

Diagram 1. Overview of the Data Interpretation Procedure within an Individual Run. Diagram from OECD 456 Test Guidelines.

[a] Conduct second or third confirmatory runs as specified in Table 1.

[b] 1.5-fold applies to both induction and inhibition of hormones.

Table 1. Decision Matrix for Possible Outcome Scenarios. Table adapted from OECD 456 Test Guidelines.

Run 1		Run 2		Run 3		Final Call
Scenario	*Decision*	*Scenario*	*Decision*	*Scenario*	*Decision*	
Negative	Confirm[a]	Negative	Stop			Negative
Negative	Confirm[a]	Positive/Equivocal[c]	Refine[b]	Negative	Stop	Negative
Negative	Confirm[a]	Positive	Refine[b]	Positive	Stop	Positive
Negative	Confirm[a]	Positive	Refine[b]	Equivocal[c]	Stop	Weak Positive
Negative	Confirm[a]	Equivocal[c]	Refine[b]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Negative	Stop	Negative
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Equivocal[c]	Confirm[a]	Equivocal[c]/Positive	Stop	Weak Positive
Equivocal[c]	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Negative	Confirm[a]	Positive	Stop	Positive

[a] Confirm previous run using the same experimental design/concentration-spacing.

[b] Re-run assay at ½ log concentration spacing (bracketing the concentration that tested significantly different in the preceding experiment). A smaller concentration-spacing in runs 2 and 3 is recommended given the chance of having only one concentration showing a significant response at a 10-fold increment. Applying a ½ log or even smaller spacing increment should result in at least two significant responses. If the confirmatory run at lower concentrations at the ½ log scale is positive, then the test article is positive. However, on rare occasions an "all-or-nothing" response may occur that only shows an effect at one concentration, which would be captured by this decision process.

[c] Fold-change at one concentration is statistically different from the solvent control.

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To evaluate the relative increase/decrease in test article altered hormone production, the results are expressed as the average fold change relative to the solvent control of each test plate. Relative changes were calculated as follows:

$$Relative\ Change = \frac{Hormone\ Concentration_{Sample}}{Hormone\ Concentration_{DMSO}}$$

A test article is judged to be positive if the fold induction or inhibition is statistically different ($p \leq 0.05$) and above or below the 1.5-fold induction or inhibition threshold from the solvent control at two adjacent concentrations in at least two independent runs (Table 1). The 1.5-fold threshold applies for both increase and decrease of hormone concentrations, i.e. above 150% of the solvent control and below 66.7% of the solvent control (where cytotoxicity ≥ 80%). A test article is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in Table 1, the experimental results are not interpretable. Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity were evaluated and passed. Statistical significance was accepted at $p \leq 0.05$.

Results

The data obtained from this study met criteria for valid assays and the positive, negative, and solvent controls performed as anticipated. AC AlfalfaBoost was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

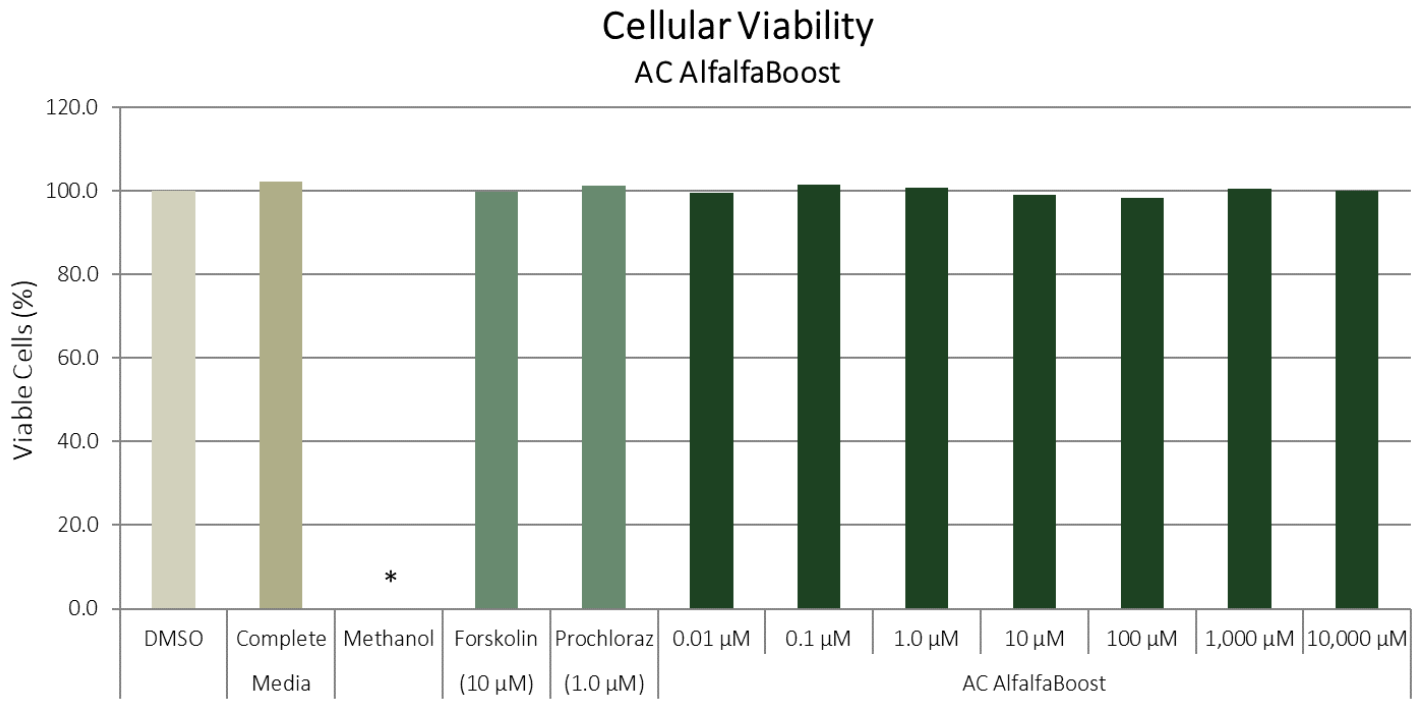

Figure 1. Cellular viability. * indicates significance ($p \leq 0.05$) compared to DMSO.

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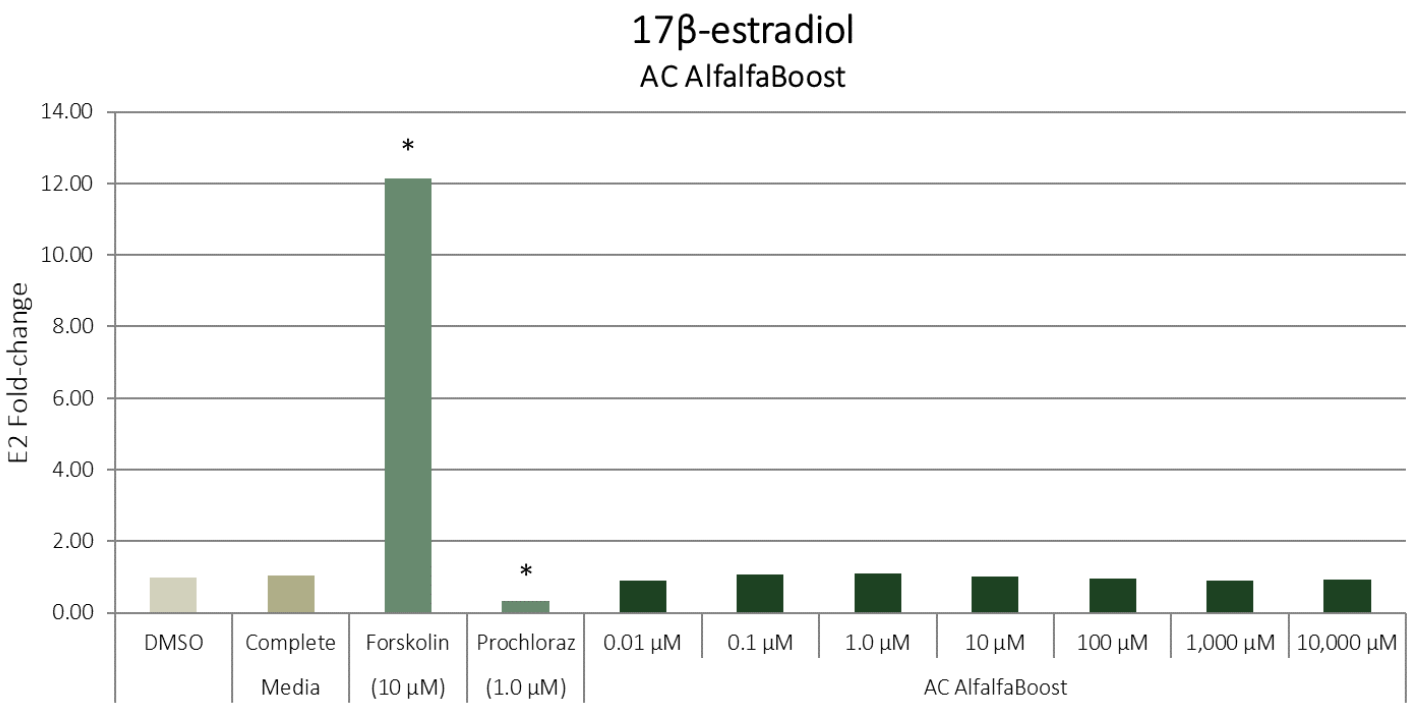

Figure 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		1.03
Forskolin		12.15*
Prochloraz		0.33*
AC AlfalfaBoost	0.01 µM	0.91
	0.1 µM	1.06
	1.0 µM	1.11
	10 µM	1.01
	100 µM	0.95
	1,000 µM	0.91
	10,000 µM	0.92

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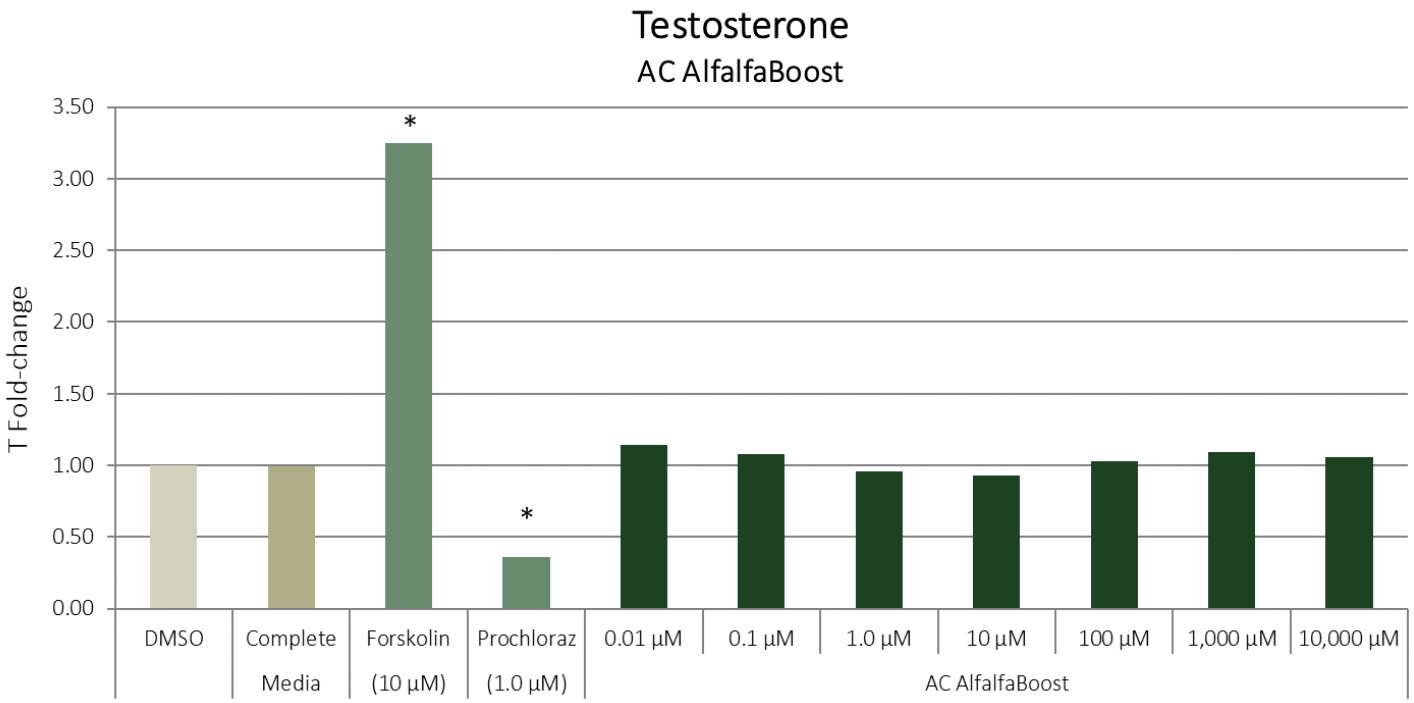

Figure 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		0.99
Forskolin		3.25*
Prochloraz		0.36*
AC AlfalfaBoost	0.01 µM	1.14
	0.1 µM	1.08
	1.0 µM	0.96
	10 µM	0.93
	100 µM	1.03
	1,000 µM	1.09
	10,000 µM	1.06

Discussion

The objective of the H295R Steroidogenesis Assay is to screen for test article effects on cellular viability and steroidogenesis by detecting substances that affect 17β-estradiol and testosterone production. Collectively, the results demonstrate **AC AlfalfaBoost** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

COMPOSITIONAL BREAKDOWN

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Leucidal® Liquid PT
Code: M15021

Compositional Breakdown:

Ingredient	%
Lactobacillus Ferment	100.00

*Please note: Leucidal® Liquid PT may contain up to 10% water soluble undecylenates.

made with Lactobacillus acidophillus
suggested use level 2%

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Active Micro Technologies hereby confirms that to the best of our knowledge, none of the potential 26 fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2

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Chemical/INCI NAME	CAS NUMBER
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: Leucidal® Liquid PT contains 100% Lactobacillus Ferment

Code: M15021

CAS #: 1686112-36-6 (or) 9015-54-7

Test Request Form #: 1421

Lot #: 40974P

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 contact[1]. 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 dermatitis[2]. 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 PT** 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.

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OECD TG 442C: *In Chemico* Skin Sensitization

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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-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-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 PT** 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
• 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)	• 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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OECD TG 442C:
In Chemico Skin Sensitization

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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±0.05mM 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±0.05mM.

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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:

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

Based on HPLC-UV analysis of **Leucidal® Liquid PT (code M15021)** 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 2.43% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD TG 442D: *In Vitro* Skin Sensitization

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Tradename: Leucidal® Liquid PT contains 100% Lactobacillus Ferment

Code: M15021

CAS #: 1686112-36-6 (or) 9015-54-7

Test Request Form #: 1422

Lot #: 40974P

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 Chemicals[1]. 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 PT** 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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OECD TG 442D: *In Vitro* Skin Sensitization

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 PT** 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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 EC1.5 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 Imax 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 EC1.5 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 PT	Non-Sensitizer	No Induction	> 1000 µM	0.6

Table 1: Overview of KeratinoSens™ Assay Results

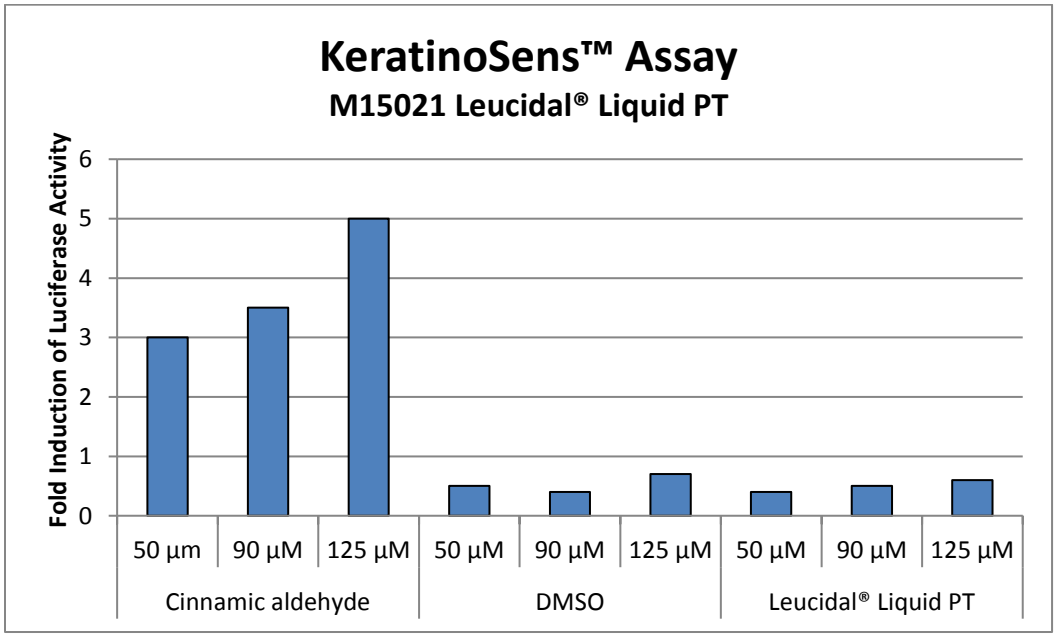

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Leucidal® Liquid PT (code M15021)** 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 PT** can be safely used in cosmetics and personal care products at typical use levels.

Bacterial Reverse Mutation Test

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107 Technology Drive • Lincolnton, NC 28092
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Test Article: Leucidal® Liquid PT
Code Number: M15021
CAS #: 68333-16-4

contains 100% Lactobacillus Ferment

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: 1020

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 PT** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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Bacterial Reverse Mutation Test

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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* WP2uvrA 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.

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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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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.

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B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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* WP2*uvr*A.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.

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Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	26	32	26
	1500	22	25	24
	500	28	36	32
	150	39	31	35
	50	25	27	26
	15	21	20	21
	5.0	32	31	30
	1.5	30	22	26
Test Solution w/o S9	5000	15	16	16
	1500	18	21	20
	500	11	19	16
	150	16	18	17
	50	17	16	17
	15	22	25	24
	5.0	20	21	21
	1.5	16	18	17
DI Water w/S9		34	41	38
DI Water w/o S9		21	27	24
2-aminoanthracen w/ S9		398	376	387
2-nitrofluorene w/o S9		245	211	228
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	115	125	120
	1500	106	112	109
	500	121	116	119
	150	145	132	139
	50	152	157	155
	15	132	155	144
	5.0	133	142	138
	1.5	138	136	137
Test Solution w/o S9	5000	133	145	139
	1500	115	125	120
	500	132	126	129
	150	117	105	111
	50	110	98	104
	15	125	125	125
	5.0	140	131	136
	1.5	141	125	133
DI Water w/S9		152	165	159
DI Water w/o S9		132	155	144
2-aminoanthracen w/ S9		402	409	406
Sodium azide w/o S9		378	399	389
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	8	8	8
	1500	12	15	14
	500	13	11	12
	150	19	17	18
	50	18	15	17
	15	12	12	12
	5.0	13	15	14
	1.5	11	5	8
Test Solution w/o S9	5000	21	22	22
	1500	25	16	21
	500	12	15	14
	150	11	12	12
	50	15	22	19
	15	16	17	17
	5.0	14	13	14
	1.5	15	8	12
DI Water w/S9		11	19	15
DI Water w/o S9		18	16	17
2-aminoanthracen w/ S9		378	355	367
2-aminoacridine w/o S9		365	389	377
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	19	18	19
	1500	26	27	27
	500	21	28	25
	150	24	20	22
	50	22	30	26
	15	21	21	21
	5.0	20	21	21
	1.5	18	25	22
Test Solution w/o S9	5000	39	42	41
	1500	38	33	36
	500	45	40	43
	150	40	21	31
	50	23	26	25
	15	25	38	32
	5.0	22	24	23
	1.5	20	26	23
DI Water w/S9		18	21	20
DI Water w/o S9		31	30	31
2-aminoanthracen w/ S9		254	288	271
Sodium azide w/o S9		381	345	363
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	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	16	16
	1500	25	28	27
	500	24	24	24
	150	28	25	27
	50	32	35	34
	15	38	31	35
	5.0	38	39	39
	1.5	41	35	38
Test Solution w/o S9	5000	32	36	34
	1500	30	41	36
	500	35	45	40
	150	18	19	19
	50	21	22	22
	15	23	24	24
	5.0	20	15	18
	1.5	41	45	43
DI Water w/S9		42	43	43
DI Water w/o S9		25	35	30
2-aminoanthracen w/ S9		484	492	488
Methylmethanesulfonate w/o S9		401	395	398
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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Dermal and Ocular Irritation Tests

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

Sample: Leucidal® Liquid PT 100% Lactobacillus Ferment

Code: M15021

CAS #: 1686112-36-6 (or) 9015-54-7

Test Request Form/Submission #: 1444

Lot #: 30371P

Sponsor: *Active Concepts, LLC; 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 PT** 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 **non-irritating**. 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% CO_2, 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-irritant**. The negative and positive controls performed as anticipated.

ACTIVE MICRO TECHNOLOGIES

Dermal and Ocular Irritation Tests

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

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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%.

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IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, 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% CO_2 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% CO_2 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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) 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.

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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 Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

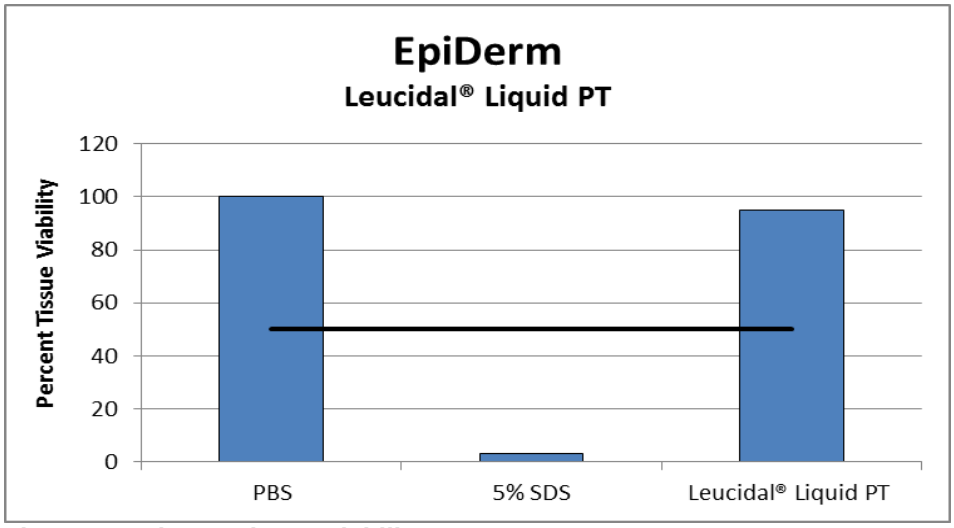

Figure 1: EpiDerm tissue viability

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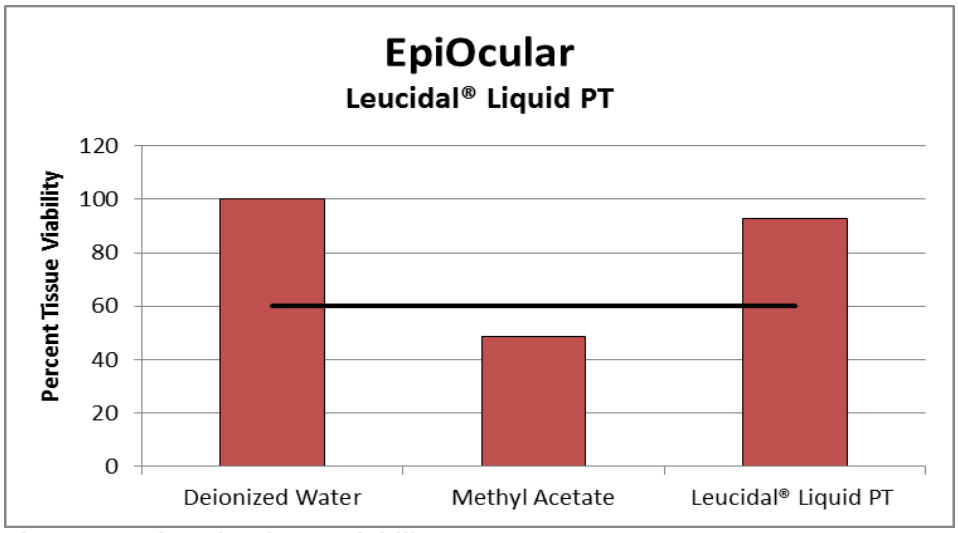

Figure 2: EpiOcular tissue viability

PRODUCT SPECIFICATION

ACTIVE MICRO TECHNOLOGIES
107 Technology Drive, Lincolnton | NC 28092 USA
info@activemicrotechnologies.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Product Name: Leucidal® Liquid PT
Code Number: M15021
CAS #'s: 1686112-36-6 (or) 9015-54-7
EINECS #'s: N/A (or) 295-635-5
INCI Name: Lactobacillus Ferment

Specification	Parameter
Appearance	Clear to Slightly Hazy Liquid
Color	Pale Yellow to Yellow
Odor	Characteristic
pH (Direct)	7.0 – 8.5
Heavy Metals	< 20 ppm
Lead	< 10 ppm
Arsenic	< 2 ppm
Cadmium	< 1 ppm

DO NOT FREEZE; Store at temperatures between 23 - 28°C;
May sediment upon standing; Mix Well Prior to Use

*Please note: Leucidal® Liquid PT may contain up to 10% water soluble undecylenates.

Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored.

Version#11/10.30.18/Form#1

COMPOSITIONAL BREAKDOWN

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107 Technology Drive, Lincolnton | NC 28092 USA
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Leucidal® Liquid SF
Code: M15019

Compositional Breakdown:

Ingredient	%
Lactobacillus Ferment	100.00

- To our knowledge the above material is free of the following list of heavy metals:
  - Heavy Metals < 20 ppm (Max.)
  - Chromium < 20 ppm (Max.)
  - Lead < 10 ppm (Max.)
  - Nickel < 10 ppm (Max.)
  - Cobalt < 10 ppm (Max.)
  - Antimony < 5 ppm (Max.)
  - Arsenic < 2 ppm (Max.)
  - Mercury < 1 ppm (Max.)
  - Cadmium < 1 ppm (Max.)

made with Lactobacillus acidophillus
suggested use level 2-4%

Version#10/10.16.23/Form#4

COMPOSITIONAL BREAKDOWN

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Active Micro Technologies hereby confirms that to the best of our knowledge, none of the potential 26 fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2

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Chemical/INCI NAME	CAS NUMBER
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Leucidal® Liquid SF Manufacturing Flow Chart

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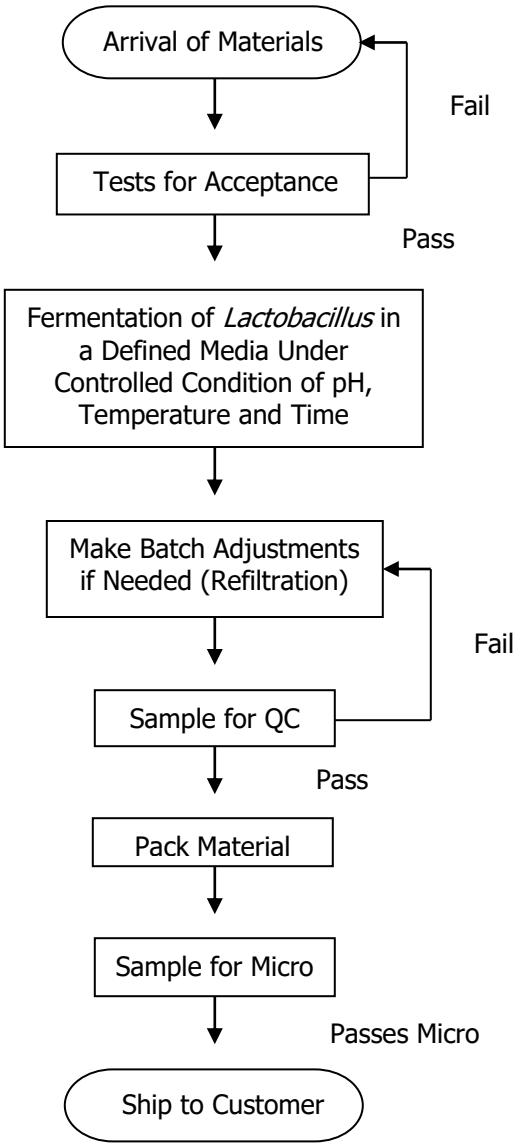

Version#1/09-27-16

OECD 456: H295R STEROIDOGENESIS ASSAY

ACTIVE MICRO TECHNOLOGIES
107 Technology Drive, Lincolnton | NC 28092 USA
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Tradename: Leucidal® Liquid SF 100% Lactobacillus Ferment

Code: M15019

CAS #: 1686112-36-6 (or) 68333-16-4

Test Request Form #: 12079

Lot #: 9401475

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
OECD 456: H295R Steroidogenesis Assay

Introduction

Endocrine disrupting agents are substances that interfere with the endocrine system and are associated with several negative health effects. Located throughout the body, endocrine glands release hormones into circulation and these signaling molecules are vital to maintaining homeostasis. Endocrine glands and hormones are extremely sensitive and small disruptions to the structure or function of any aspect of the endocrine system significantly alter physiological processes. Endocrine disruptors work through one or a combination of the following mechanisms: 1) mimicking, or partly mimicking, naturally occurring hormones, potentially leading to over production, 2) binding to a receptor and blocking a naturally occurring hormone from binding, and/or 3) interfering with or preventing the synthesis or utilization of natural hormones and/or receptors. The OECD 456: H295R Steroidogenesis Assay was developed as an *in vitro* test to screen for test article effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T).

A H295R Steroidogenesis Assay was conducted to determine the potential of **Leucidal® Liquid SF** to induce or inhibit the production of 17β-estradiol and testosterone.

Assay Principle

The *in vitro* H295R Steroidogenesis Assay utilizes a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 "*in vitro* assay, providing mechanistic data" to screen for test article effects on steroidogenesis, but does not aim to provide specific mechanistic information concerning the interaction of the test substance with the endocrine system. Moreover, the H295R assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic pituitary-gonadal axis. The objective of the H295R Steroidogenesis Assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone (T). The H295R assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions from cholesterol to the production of E2 and/or T. Human adreno-carcinoma H295R cells are utilized because the cell line expresses genes that encode all key steroidogenesis enzymes, enables the detection of both increases and decreases in the production of both E2 and T, and permits the direct assessment of the potential impact of a test article on cell viability/cytotoxicity. The purpose of the assay is the detection of substances that affect E2 and T production by distinguishing test articles that typically inhibit or induce key enzymes of the steroidogenesis pathway.

OECD 456: H295R STEROIDOGENESIS ASSAY

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The human adreno-carcinoma H295R cells are exposed to different concentrations of the test substance. After the incubation period, the medium is removed from each well and cellular viability is immediately analyzed. Next, concentrations of E2 and T in the medium are measured.

Materials

A. Kit:	Estradiol ELISA Kit (Cayman Chemical; 501890)*; DetectX® Testosterone ELISA Kit (Arbor Assays; K032-H)*; CyQUANT™ MTT Cell Viability Assay (ThermoFisher; V13154)*
B. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
C. Equipment:	Forma Humidified Incubator; ESCO Biosafety Laminar Flow Hood; Synergy HT Microplate Reader; Pipettes; Light Microscope; Weigh Boats; Chemical Spatula; Analytical Balance
D. Cell Line:	Human Adreno-Carcinoma Cell (NCI-H295R [H295R]) (ATCC; CRL-2128)*
E. Media/Buffers:	DMEM: F-12 Medium (ATCC; 30-2006)*; ITS+ Premix Universal Culture Supplement (Corning®; 354352)*; Nu-Serum™ Growth Medium Supplement (Corning®; 355100)*; Dimethyl sulfoxide (DMSO); Deionized water
F. Reagents:	Forskolin (ThermoFisher; J63292.MF)*; Prochloraz (ThermoFisher; P21371G)* Phosphate Buffered Saline (PBS); Trypsin; Trypsin-EDTA; Methanol; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT Assay; ThermoFisher; V13154); SDS-HCl Solution (sodium dodecyl sulfate hydrochloride) (MTT Assay; ThermoFisher; V13154)
G. Culture Plate:	24 Well Tissue Culture Treated Microplates; Tissue Culture Treated Flasks
H. Software:	Excel Analysis ToolPak (Microsoft)
I. Other:	Sterile disposable pipette tips

**Or suitable alternatives, subject to change without notice based off vendor availability*

Methods

One source vial of H295R cells were initiated from cryopreservation and expanded three passages to establish a lot for screening and cells were expanded five times before used for testing or frozen for storage. Cells cultured from the expanded stock did not exceed 10 passages. Cells were passaged and maintained in Complete Media (DMEM: F-12 Medium + 1.0% ITS+ Premix Universal Culture Supplement + 2.5% Nu-Serum™ Growth Medium Supplement). The maintenance, expansion, and cryopreservation standard operating procedures were adopted directly from the guideline specifications. A quality control test plate to establish acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement assays, and variability between replicate hormone measures of the same sample were performed and met criteria according to the guideline specifications.

Cells utilized for testing were seeded at a target density of 200,000 to 300,000 cells per mL of Complete Media resulting in approximately 50-60% confluency in the wells at 24 hours to achieve the optimal cell density for hormone production. After an acclimation period of 24 hours, cells were exposed for 48 hours to seven concentrations of **Leucidal® Liquid SF**, diluted with DMSO, in triplicate. Cells were also exposed to Complete Media and positive, negative, and solvent controls. Prepared in DMSO, Forskolin (10 µM) and Prochloraz (1.0 µM) were utilized as positive and negative controls as they are known inducers and inhibitors, respectively, of E2 and T synthesis. DMSO was utilized as the solvent control. At the end of the 48-hour exposure period, the media is removed from each well and stored in aliquots at -80°C until hormone analysis. Cell viability in each well is analyzed immediately after removal of media. Conclusions regarding the ability of a test article to affect steroidogenesis are based on at least two independent experimental runs.

Version#1/05.31.24/Form#267

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To determine cell viability, fresh Complete Media with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to all wells, except for the positive control wells which received 70% Methanol in Complete Media provided Methanol is a known cytotoxicity inducer. After a 4-hour incubation period at 37°C, the SDS-HCl solution was added to all wells and the plate was incubated again at 37°C for 4 hours after which the absorbance was read at 570 nm. Cell viability data is displayed as Mean Absorbance Units (MAU) and reflects averages of the triplicates for independent experimental runs. The results were analyzed utilizing a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Conditions demonstrating viability lower than 80% were not included in the final data analysis. The percentage of viable cells was calculated with the following equation:

$$Viable\ Cells\ (\%) = \frac{MAU_{Sample} - MAU_{Methanol}}{MAU_{DMSO} - MAU_{Methanol}} \times 100$$

After cellular viability was determined, 17β-estradiol (E2) and testosterone (T) concentration levels in the media were determined with an Estradiol ELISA Kit (Limit of Quantification 6.0 pg/mL) and DetectX® Testosterone ELISA Kit (Limit of Quantification 30.6 pg/mL), respectively. Frozen media aliquots were thawed at room temperature and both ELISAs were performed according to the manufacturers' protocols. Briefly, thawed media aliquots were loaded onto the plate along with prepared standards. Next, conjugates and antibodies were added to appropriate wells and allowed to incubate at room temperature. After the incubation, wells were washed, and antibody detection reagents were added to each well followed by stop solutions to stop the antibody detection reactions. Lastly, absorbance values were measured.

The criteria for a negative, positive, or equivocal run are provided in Diagram 1. A minimum of two independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at $\log_{10}$ intervals with 10^{-3} M being the maximum concentration. If the test article is soluble, and not cytotoxic at any of the tested concentrations, and the first run was negative for all concentrations, then it is to be confirmed in one more run using the same conditions as the first run was conducted (Table 1). If the results of the first run are equivocal or positive, the test should be repeated as indicated in Table 1 and footnote 'a' to Diagram 1, by refining the selected test concentrations. Test concentrations in runs two and three (if applicable) should be adjusted on the basis of the results of the initial run bracketing concentrations that elicited an effect using ½-log concentration spacing (e.g. if the original run of 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM resulted in inductions at 1 and 10 µM, the concentrations tested in the second run should be 0.1, 0.3, 1, 3, 10, 30, 100 µM), unless lower concentrations need to be employed to achieve a Lowest-Observed-Effect-Concentration (LOEC). In the latter case, at least five concentrations below the lowest concentration tested in the first run should be used in the second run using a ½-log scale. If the assay is equivocal or positive, the LOEC is reported. If the assay is negative, the highest concentration tested is reported as the No-Observed-Effect-Concentration (NOEC).

If the second run does not confirm the first run, a third experiment is to be conducted using the original testing conditions. Equivocal results in the first run are considered negative if the observed effect could not be confirmed in any of the two subsequent runs. Equivocal results are considered as weak positive responses (effect) when the response can be confirmed in at least two more runs within a ± 1 concentration increment (Diagram 1; Table 1). Non monotonic concentration response patterns are rare but possible. If reproducible effects are seen at lower concentrations which then dissipate at greater concentrations and/or return at the top concentrations, this data should not be disregarded and should be included in the study report.

OECD 456: H295R STEROIDOGENESIS ASSAY

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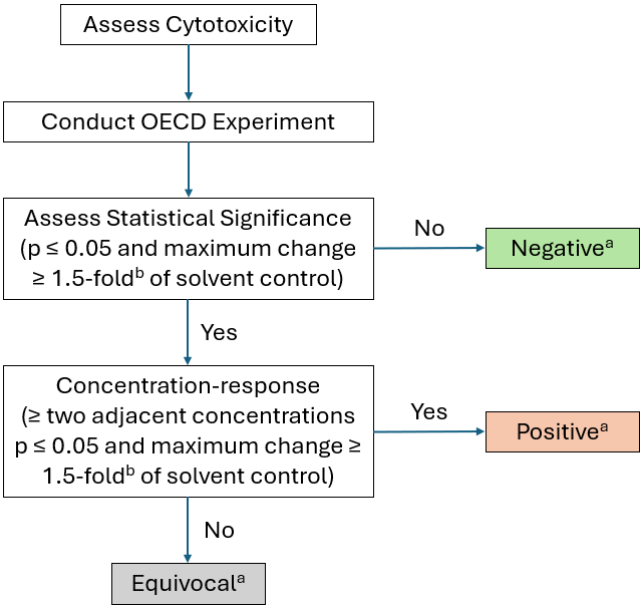

Diagram 1. Overview of the Data Interpretation Procedure within an Individual Run. Diagram from OECD 456 Test Guidelines.

[a] Conduct second or third confirmatory runs as specified in Table 1.

[b] 1.5-fold applies to both induction and inhibition of hormones.

Table 1. Decision Matrix for Possible Outcome Scenarios. Table adapted from OECD 456 Test Guidelines.

Run 1		Run 2		Run 3		Final Call
Scenario	*Decision*	*Scenario*	*Decision*	*Scenario*	*Decision*	
Negative	Confirm[a]	Negative	Stop			Negative
Negative	Confirm[a]	Positive/Equivocal[c]	Refine[b]	Negative	Stop	Negative
Negative	Confirm[a]	Positive	Refine[b]	Positive	Stop	Positive
Negative	Confirm[a]	Positive	Refine[b]	Equivocal[c]	Stop	Weak Positive
Negative	Confirm[a]	Equivocal[c]	Refine[b]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Negative	Stop	Negative
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Equivocal[c]	Confirm[a]	Equivocal[c]/Positive	Stop	Weak Positive
Equivocal[c]	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Negative	Confirm[a]	Positive	Stop	Positive

[a] Confirm previous run using the same experimental design/concentration-spacing.

[b] Re-run assay at ½ log concentration spacing (bracketing the concentration that tested significantly different in the preceding experiment). A smaller concentration-spacing in runs 2 and 3 is recommended given the chance of having only one concentration showing a significant response at a 10-fold increment. Applying a ½ log or even smaller spacing increment should result in at least two significant responses. If the confirmatory run at lower concentrations at the ½ log scale is positive, then the test article is positive. However, on rare occasions an "all-or-nothing" response may occur that only shows an effect at one concentration, which would be captured by this decision process.

[c] Fold-change at one concentration is statistically different from the solvent control.

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To evaluate the relative increase/decrease in test article altered hormone production, the results are expressed as the average fold change relative to the solvent control of each test plate. Relative changes were calculated as follows:

$$Relative\ Change = \frac{Hormone\ Concentration_{Sample}}{Hormone\ Concentration_{DMSO}}$$

A test article is judged to be positive if the fold induction or inhibition is statistically different ($p \leq 0.05$) and above or below the 1.5-fold induction or inhibition threshold from the solvent control at two adjacent concentrations in at least two independent runs (Table 1). The 1.5-fold threshold applies for both increase and decrease of hormone concentrations, i.e. above 150% of the solvent control and below 66.7% of the solvent control (where cytotoxicity ≥ 80%). A test article is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in Table 1, the experimental results are not interpretable. Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity were evaluated and passed. Statistical significance was accepted at $p \leq 0.05$.

Results

The data obtained from this study met criteria for valid assays and the positive, negative, and solvent controls performed as anticipated. **Leucidal® Liquid SF** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

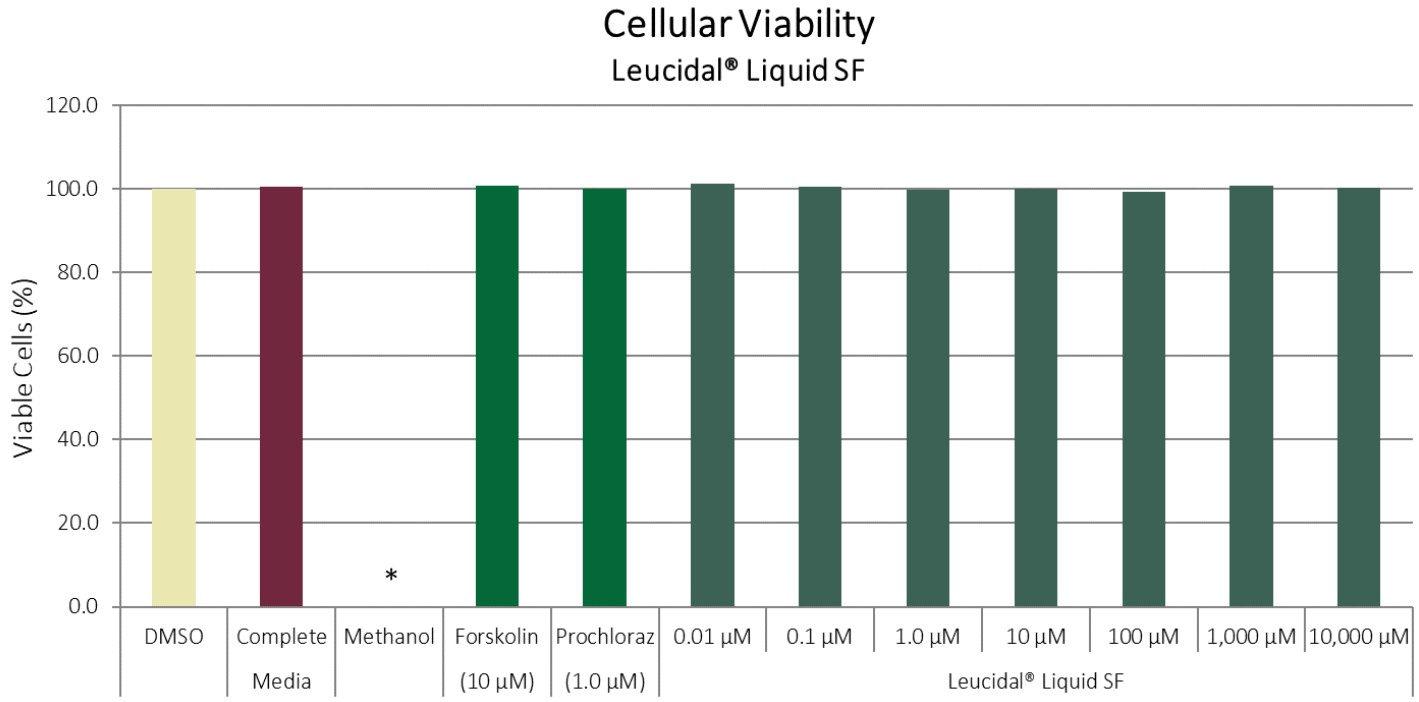

Figure 1. Cellular viability. * indicates significance ($p \leq 0.05$) compared to DMSO.

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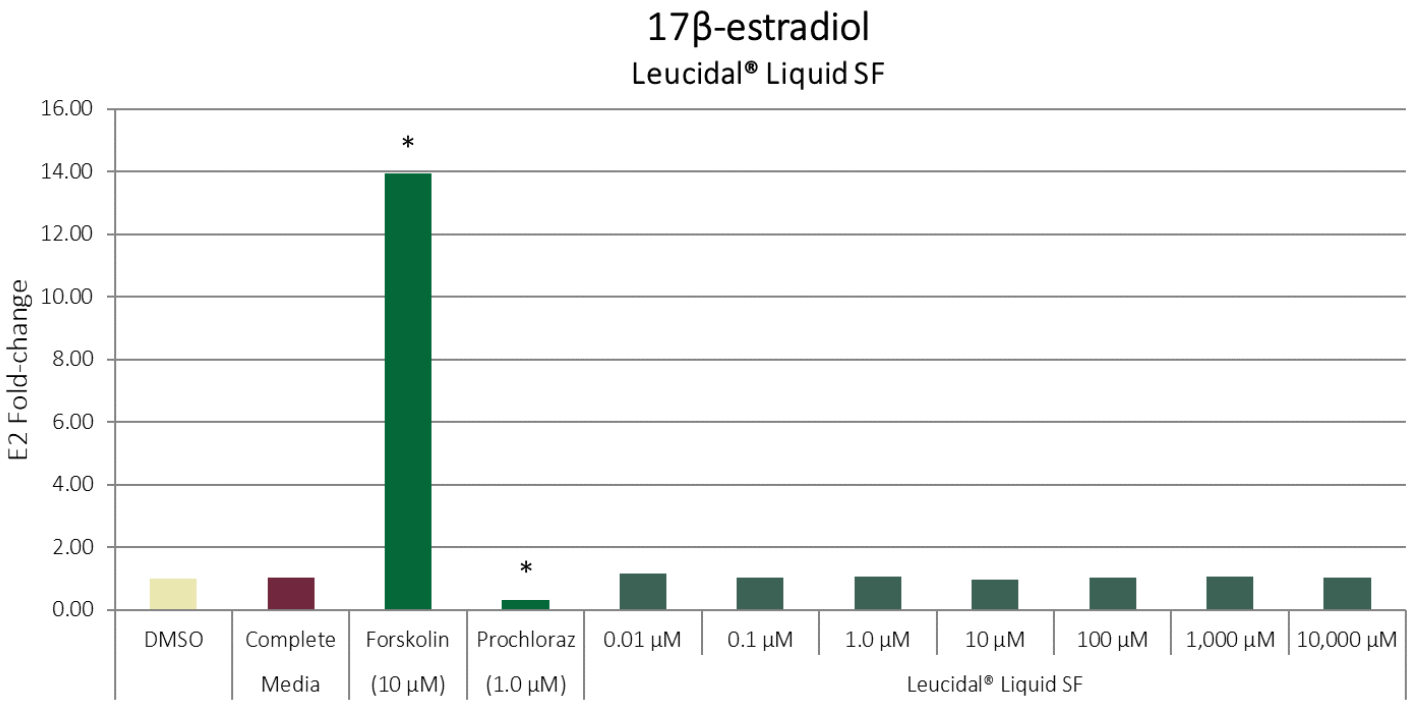

Figure 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		1.03
Forskolin		13.94*
Prochloraz		0.31*
Leucidal® Liquid SF	0.01 µM	1.15
	0.1 µM	1.03
	1.0 µM	1.07
	10 µM	0.96
	100 µM	1.02
	1,000 µM	1.07
	10,000 µM	1.04

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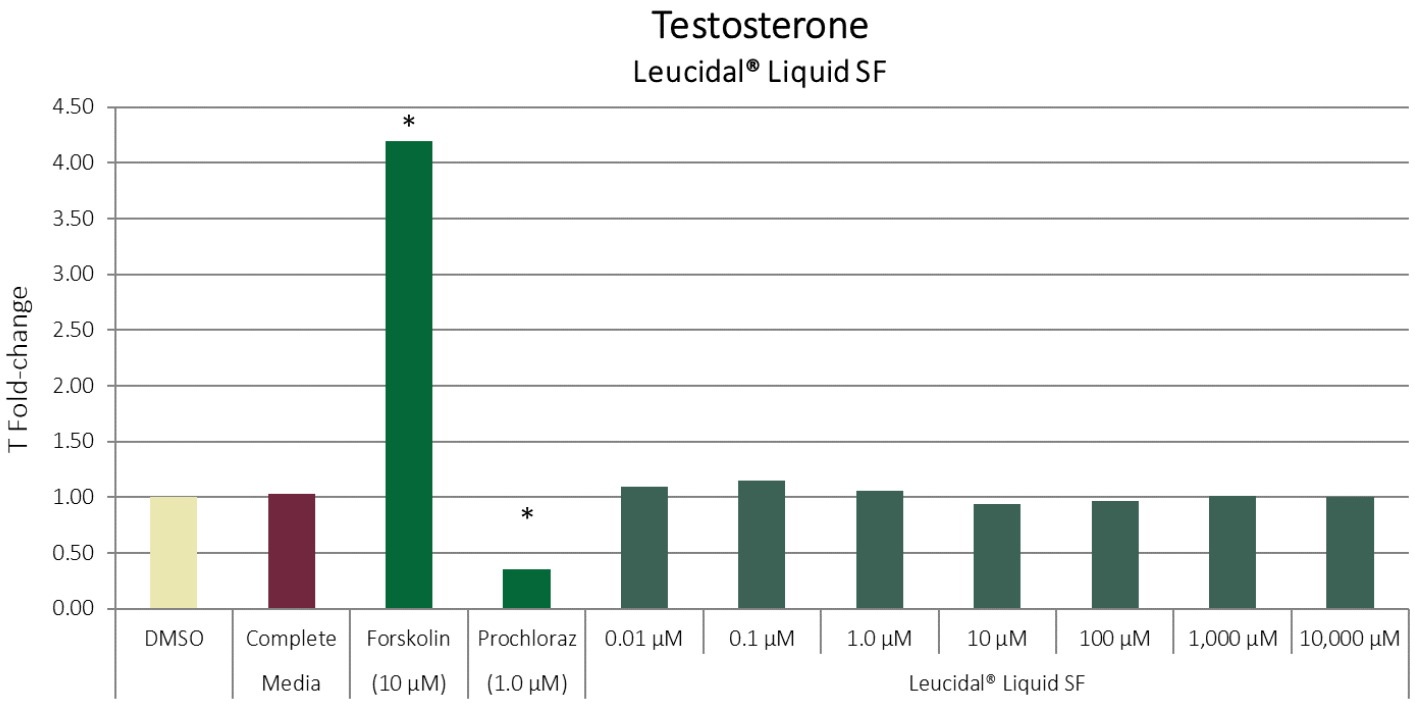

Figure 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		1.03
Forskolin		4.19*
Prochloraz		0.35*
Leucidal® Liquid SF	0.01 µM	1.09
	0.1 µM	1.15
	1.0 µM	1.06
	10 µM	0.94
	100 µM	0.97
	1,000 µM	1.01
	10,000 µM	1.00

Discussion

The objective of the H295R Steroidogenesis Assay is to screen for test article effects on cellular viability and steroidogenesis by detecting substances that affect 17β-estradiol and testosterone production. Collectively, the results demonstrate **Leucidal® Liquid SF** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: Leucidal® Liquid SF 100% Lactobacillus Ferment

Code: M15019

CAS #: 1686112-36-6 (or) 68333-16-4

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 contact[1]. 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 dermatitis[2]. 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.

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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-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-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
• 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)	• 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±0.05mM 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±0.05mM.

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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

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.43	Minimal Reactivity	Non-sensitizer
3.46	Minimal Reactivity	Non-sensitizer
3.44	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.60	Minimal Reactivity	Non-sensitizer
3.58	Minimal Reactivity	Non-sensitizer
3.57	Minimal Reactivity	Non-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:

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

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. Both Cysteine and Lysine prediction models fall below 6.38% that is the upper threshold for mean percent depletion for the minimal reactivity class predicting a non-sensitizer. 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. All values for the standard calibration curve and mean percent peptide depletion for the positive control were met.

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OECD TG 442D: *In Vitro* Skin Sensitization

Tradename: Leucidal® Liquid SF 100% Lactobacillus Ferment

Code: M15019

CAS #: 1686112-36-6

Test Request Form #: 4754

Lot #: 5622P

Sponsor: *Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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 Chemicals[1]. 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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.

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In Vitro Skin Sensitization

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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 EC1.5 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.

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

1. The Imax 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 EC1.5 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

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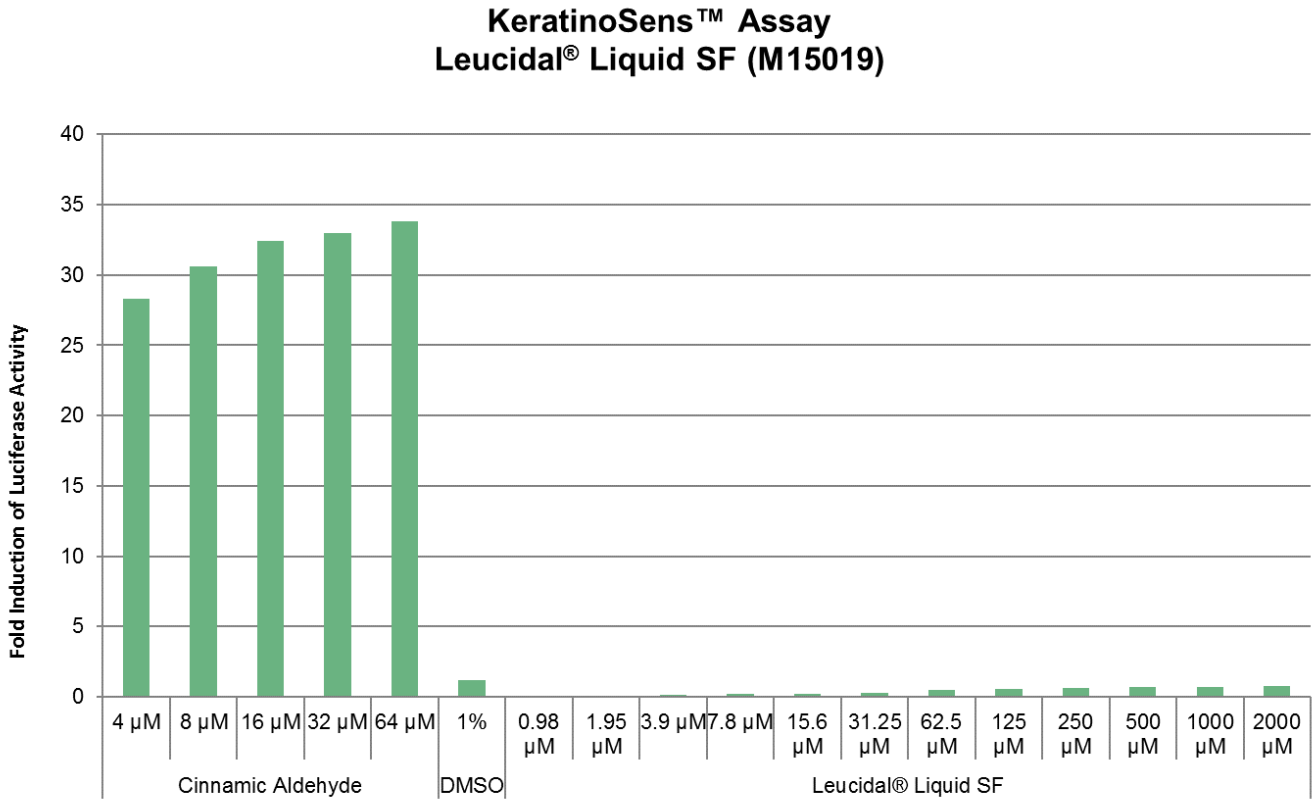

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Leucidal® Liquid SF (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.

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Phototoxicity Assay Analysis

Tradename: Leucidal® Liquid SF 100% Lactobacillus Ferment

Code: M15019

CAS #: 1686112-36-6 (or) 68333-16-4

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% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). 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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Phototoxicity Assay Analysis

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II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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 chloropromazine, 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/cm^2 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% CO_2 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% CO_2, 95% RH).

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C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 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% CO_2, 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% CO_2, 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 (OD_{570}) 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.

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Phototoxicity Assay Analysis

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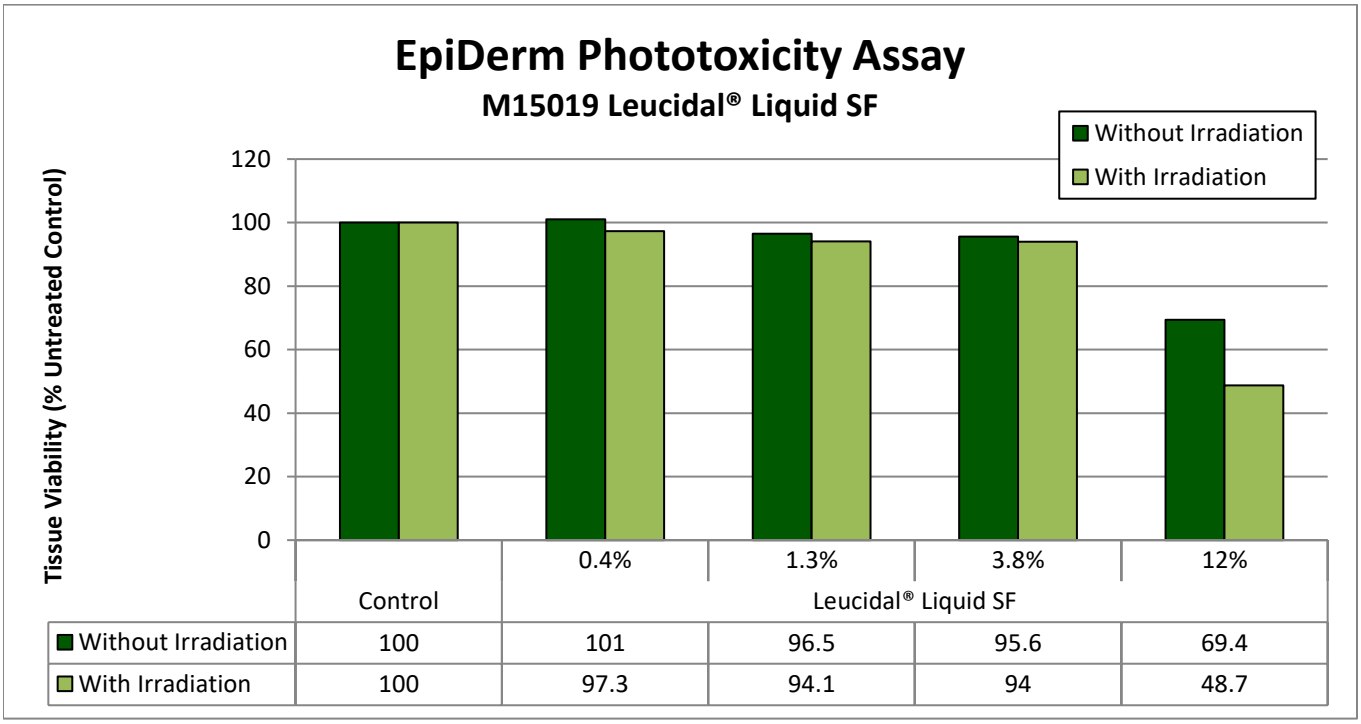
VII. Conclusion

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%.



	Control	0.4%	1.3%	3.8%	12%
Without Irradiation	100	101	96.5	95.6	69.4
With Irradiation	100	97.3	94.1	94	48.7

Figure 1: EpiDerm Phototoxicity Graph

ACTIVE MICRO TECHNOLOGIES

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The following report evaluates a sample of

Leucidal® Liquid SF (M15019) – AMA Lab No. N-6324
100% Lactobacillus Ferment
provided by Active Micro Technologies, LLC to AMA Laboratories, Inc.

Utilizing the Repeat Insult Patch Test
Skin Irritation / Sensitization Evaluation (Occlusive Patch)

October 13, 2014

AMA LABORATORIES, INC.
216 Congers Road, Bldg. 1
New City, NY 10956 USA
(845) 634-4330
FAX: (845) 634-5565
www.amalabs.com

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	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	Chall. 48 HR	Score
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	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	Chall. 48 HR	Score
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:

Tasmiya Masud, B.A.
Quality Assurance Supervisor

10/13/14
Date

Bacterial Reverse Mutation Test

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

Test Article: Leucidal® Liquid SF
Code Number: M15019
CAS #: 1686112-36-6 (or) 68333-16-4

100% Lactobacillus Ferment

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 reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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Bacterial Reverse Mutation Test

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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* WP2uvrA 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.

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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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

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C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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* WP2*uvr*A.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.

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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	***WP2uvrA***		
		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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Dermal and Ocular Irritation Tests

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Sample: Leucidal® Liquid SF 100% Lactobacillus Ferment

Code: M15019

CAS #: 1686112-36-6 (or) 68333-16-4

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% CO_2, 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.

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Dermal and Ocular Irritation Tests

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II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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% CO_2 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% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) 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.

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

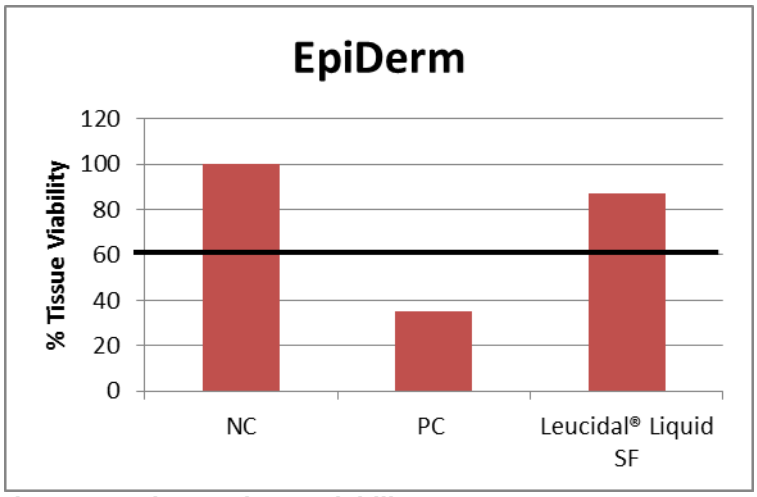

Figure 1: EpiDerm tissue viability

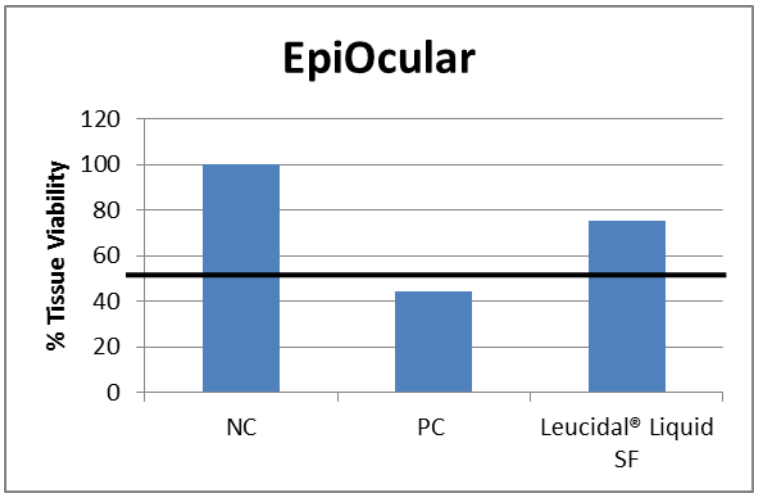

Figure 2: EpiOcular tissue viability

PRODUCT SPECIFICATION

ACTIVE MICRO TECHNOLOGIES
107 Technology Drive, Lincolnton | NC 28092 USA
info@activemicrotechnologies.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

US Patent Number 10,232,197

Product Name: Leucidal® Liquid SF
Code Number: M15019
CAS #'s: 1686112-36-6 (or) 68333-16-4
EINECS #'s: N/A (or) 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
Lead	< 10 ppm
Arsenic	< 2 ppm
Cadmium	< 1 ppm
Bacteriocins (HPLC)	1.00 – 10.00%
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

Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored.

Note:
1) Refer to Inhibition Activity Data

Version#19/08.22.19/Form#1

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Compositional Breakdown

Leucidal® SF Max
Code: M15019MAX

Compositional Breakdown:

Ingredient	%
Lactobacillus Ferment	100.00

- **To our knowledge the above material is free of the following list of heavy metals:**
  - **Heavy Metals < 20 ppm (Max.)**
  - **Chromium < 20 ppm (Max.)**
  - **Lead < 10 ppm (Max.)**
  - **Nickel < 10 ppm (Max.)**
  - **Cobalt < 10 ppm (Max.)**
  - **Antimony < 5 ppm (Max.)**
  - **Arsenic < 2 ppm (Max.)**
  - **Mercury < 1 ppm (Max.)**
  - **Cadmium < 1 ppm (Max.)**

made with Lactobacillus acidophilus
suggested use level: 2-4%

Compositional Breakdown

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Active Micro Technologies hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Micro Technologies hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

M15019MAX-Leucidal® SF Max Manufacturing Flow Chart

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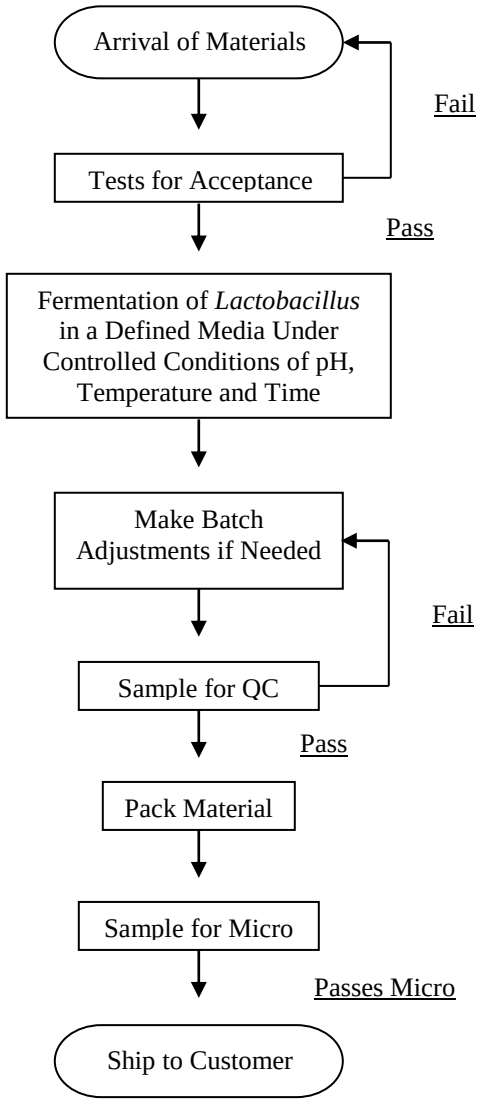

ACTIVE MICRO TECHNOLOGIES

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US Patent Number 10,232,197

Specification

Product Name: Leucidal® SF Max
Code Number: M15019MAX
CAS #'s: 1686112-36-6 (or) 68333-16-4
EINECS #'s: N/A (or) N/A
INCI Name: Lactobacillus Ferment

Specification	Parameter
Appearance	Clear to Slightly Hazy Liquid
Color	Colorless to Yellow
Odor	Characteristic
Solids (1g/1hr/105°C)	20.0 – 25.0%
pH (Direct)	4.0 – 11.0
Heavy Metals	< 20 ppm
Lead	< 10 ppm
Arsenic	< 2 ppm
Cadmium	< 1 ppm
Bacteriocins (HPLC)	5.00 – 10.00%
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

Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored.

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US Patent Number 10,232,197

Note:

1) Refer to Inhibition Activity Data

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: Leucidal® Liquid SF Max 100% Lactobacillus Ferment

Code: M15019MAX

CAS #: 1686112-36-6 (or) 68333-16-4

Test Request Form #: 3684

Lot #: NC170915-E

Sponsor: *Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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 contact[1]. 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 dermatitis[2]. 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 Max** 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.

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OECD TG 442C:
In Chemico Skin Sensitization

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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-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-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 Max** 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
• 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)	• 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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OECD TG 442C:
In Chemico Skin Sensitization

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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±0.05mM 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±0.05mM.

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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:

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

Based on HPLC-UV analysis of **Leucidal® Liquid SF Max (code M15019MAX)** 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 3.26% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD TG 442D: *In Vitro* Skin Sensitization

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

Tradename: Leucidal® Liquid SF Max 100% Lactobacillus Ferment

Code: M15019MAX

CAS #: 1686112-36-6 (or) 68333-16-4

Test Request Form #: 3683

Lot #: NC170915-E

Sponsor: *Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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 Chemicals[1]. 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 Max** 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

ACTIVE MICRO TECHNOLOGIES

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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 Max** 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% CO_2 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% CO_2. 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 IC_{50} and IC_{30} 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 EC1.5 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.

ACTIVE MICRO TECHNOLOGIES

OECD TG 442D:
***In Vitro* Skin Sensitization**

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

1. The Imax 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 EC1.5 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	31.4
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.16
Leucidal® Liquid SF Max	Non-Sensitizer	No Induction	> 1000 µM	0.38

Table 1: Overview of KeratinoSens™ Assay Results

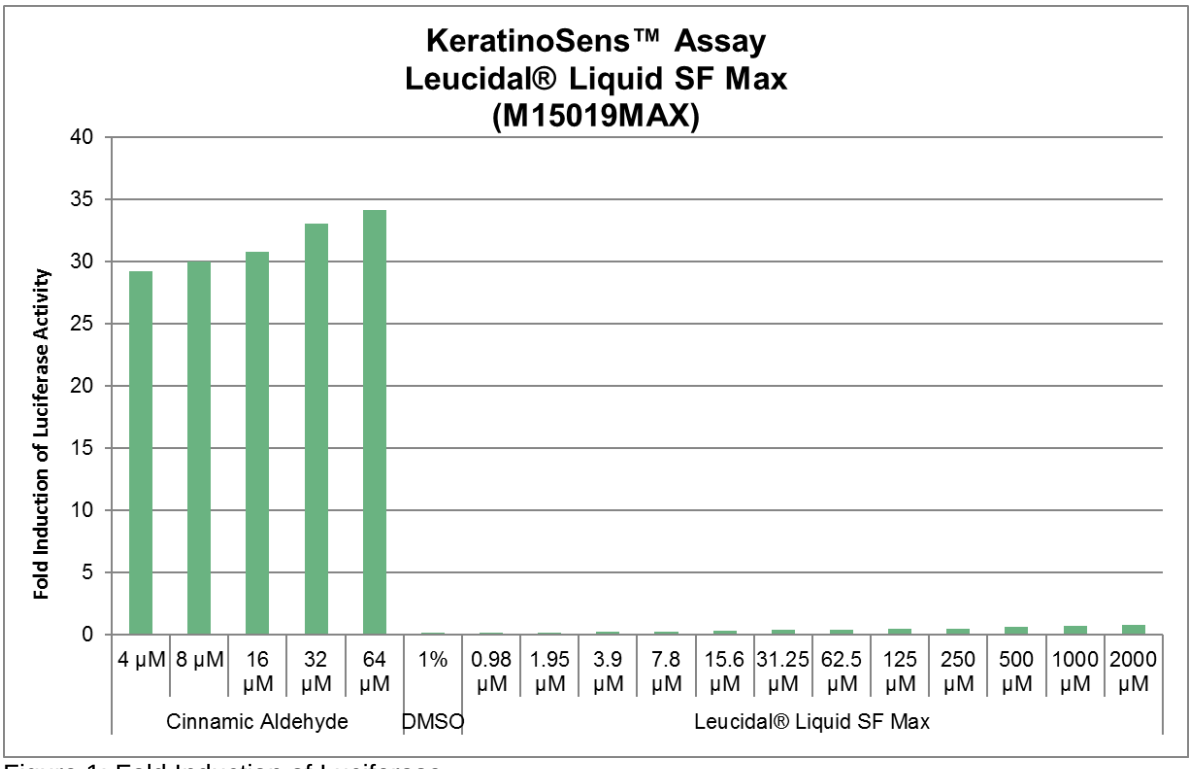

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **Leucidal® Liquid SF (code M15019MAX)** 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.

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Phototoxicity Assay Analysis

Tradename: Leucidal® SF Max 100% Lactobacillus Ferment

Code: M15019MAX

CAS #: 1686112-36-6 (or) 68333-16-4

Test Request Form #: 3687

Lot #: NC170915-E

Sponsor: *Active Micro Technologies, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **Leucidal® SF Max** 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 0.5%, 1.5%, and 5.0%. 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 four varying concentrations and incubated overnight at 37 °C, 5% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). 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 0.5%, 1.5%, and 5.0%. 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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Phototoxicity Assay Analysis

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II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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 chloropromazine, 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/cm^2 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% CO_2 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% CO_2, 95% RH).

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C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 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% CO_2, 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% CO_2, 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 (OD_{570}) 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 0.5%, 1.5%, and 5.0%. 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.

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Phototoxicity Assay Analysis

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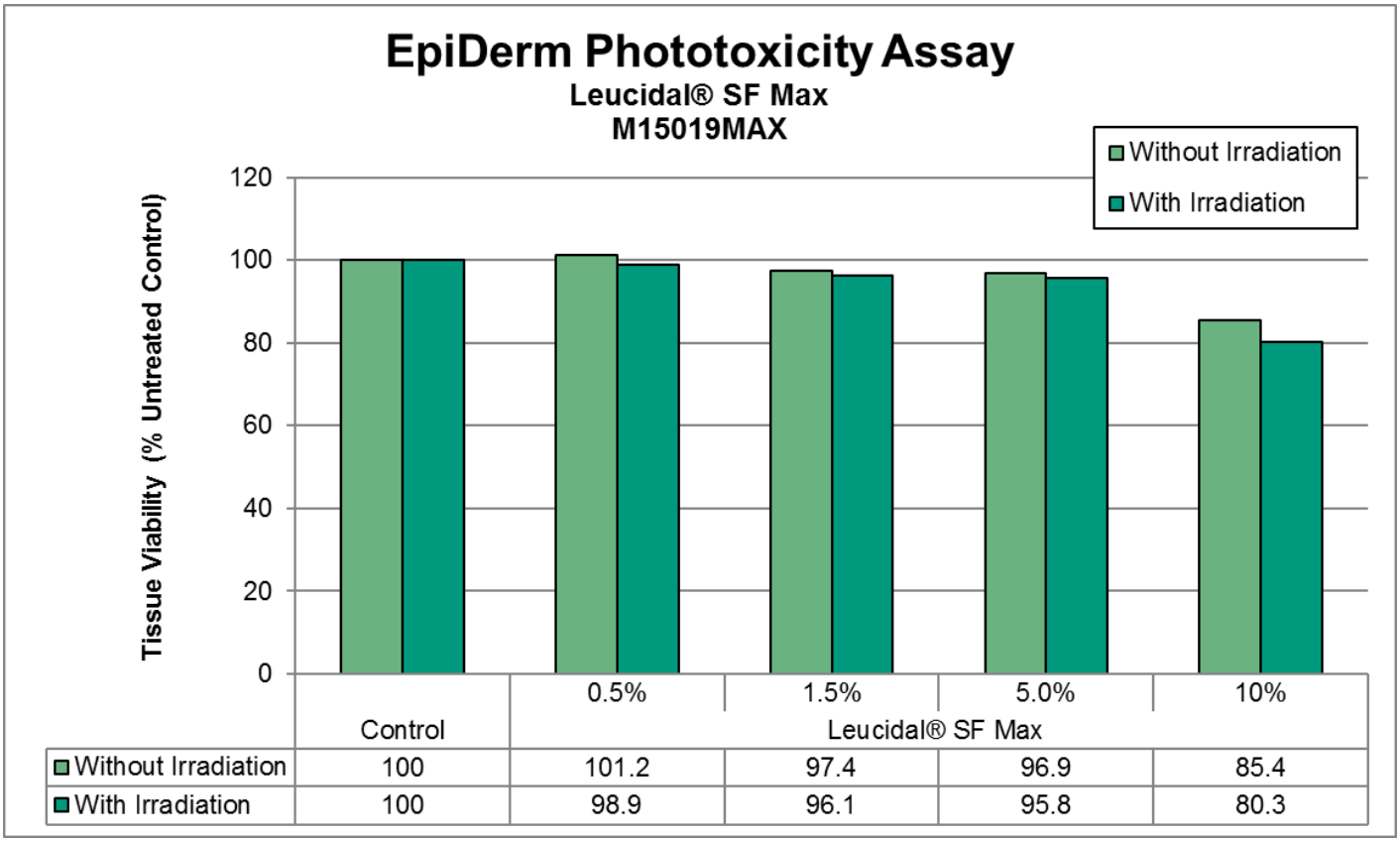
VII. Conclusion

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 0.5%, 1.5%, and 5.0%. There is a decrease in viability at the 10% 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® SF Max** is not a photoirritant when used at the suggested use levels of 2.0 – 4.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	101.2	97.4	96.9	85.4
With Irradiation	100	98.9	96.1	95.8	80.3

Figure 1: EpiDerm Phototoxicity Graph

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The following report evaluates a sample of

Leucidal® SF Max (M15019MAX) – CPT Study No. C19-2349.02
100% Lactobacillus Ferment

provided by Active Micro Technologies, LLC

to Consumer Product Testing Company

Utilizing the Repeat Insult Patch Test
Skin Irritation / Sensitization Evaluation (Occlusive Patch)

June 17, 2019

CONSUMER PRODUCT TESTING COMPANY
cptclabs.com

FINAL REPORT

CLIENT: Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

ATTENTION: Jillian Barrow

TEST: Repeated Insult Patch Test
Protocol No.: CP-01.01S
Protocol Date: 09/28/14

TEST MATERIAL: M15019MAX Leucidal® SF Max Lot: 6647

STUDY NUMBER: C19-2349.02

Reviewed by: Richard R. Eisenberg, M.D.
Medical Director
Board Certified Dermatologist

Approved by: Michael Caswell, Ph.D., CCRA, CCRC
Vice President, Clinical Evaluations

Approved by: Joy Frank, R.N.
Executive Vice President, Clinical Evaluations

FDA Registration# 1000151293
DEA Registration# RC0199744 Schedule I-V
US EPA/NJ DEP Registration# NJD982726648
ISO/IEC 17025:2005 Accredited

Office: +1 (973) 808-7111 Fax: +1 (973) 808-7234 70 New Dutch Lane Fairfield, NJ 07004-2514

CONSUMER PRODUCT TESTING COMPANY
cptclabs.com

FDA Registration# 1000151293
DEA Registration# RC0199744 Schedule I-V
US EPA/NJ DEP Registration# NJD982726648
ISO/IEC 17025 2005 Accredited

QUALITY ASSURANCE UNIT STATEMENT

Study Number: C19-2349.02

The Consumer Product Testing Company, Incorporated (CPTC) Quality Assurance Unit (QAU) is responsible for auditing the conduct, content and reporting of all clinical trials that are conducted at CPTC.

This trial has been conducted in accordance with the Declaration of Helsinki, the ICH Guideline E6 for *Good Clinical Practice*, the requirements of 21 CFR Parts 50 and 56, other applicable laws and regulations, CPTC Standard Operating Procedures, and the approved protocol.

The CPTC QAU has reviewed all data, records, and documents relating to this trial and also this Final Report. The following QAU representative signature certifies that all data, records, and documents relating to this trial and also this Final Report have been reviewed and are deemed to be acceptable, and that the trial conforms to all of the requirements as indicated above.

All records and documents pertaining to the conduct of this trial shall be retained in the CPTC archives for a minimum of ten (10) years. At any time prior to the completion of the tenth archival year, a Sponsor may submit a written request to the CPTC QAU to obtain custody of trial records once the CPTC archive period has been completed. This transfer shall be performed at the Sponsor's expense. In the absence of a written request, trial-related records shall be destroyed at the end of the CPTC archive period with no further notice in a manner that renders them useless.

William Cavaliere
Quality Assurance Representative

6/17/2019
Date

Objective: To determine by repetitive epidermal contact the potential of a test material to induce primary or cumulative irritation and/or allergic contact sensitization.

Participants: Fifty-eight (58) qualified subjects, male and female, ranging in age from 16 to 77 years, were selected for this evaluation. Fifty-one (51) subjects completed this study. The remaining subjects discontinued their participation for various reasons, none of which were related to the application of the test material.

Inclusion Criteria:

a. Male and female subjects, age 16[a] to 79 years.
b. Absence of any visible skin disease which might be confused with a skin reaction from the test material.
c. Prohibition of use of topical or systemic steroids and/or antihistamines for at least seven days prior to study initiation.
d. Completion of a Medical History Form and the understanding and signing of an Informed Consent Form.
e. Considered reliable and capable of following directions.

Exclusion Criteria:

a. Ill health.
b. Under a doctor's care or taking medication(s) which could influence the outcome of the study.
c. Females who are pregnant or nursing.
d. A history of adverse reactions to cosmetics or other personal care products.

Test Material: M15019MAX Leucidal® SF Max Lot: 6647

Study Schedule:

Panel #	Initiation Date	Completion Date
20190145	April 3, 2019	May 10, 2019

[a]With parental or guardian consent

Methodology:

The test material was received as a 10% dilution, in water.

The upper back between the scapulae served as the treatment area. Approximately 0.2 ml of the test material, or an amount sufficient to cover the contact surface, was applied to the 3/4" x 3/4" absorbent pad portion of an adhesive dressing. This was then applied to the appropriate treatment site to form an occlusive patch.

Induction Phase:

Patches were applied three (3) times per week (e.g., Monday, Wednesday, and Friday) for a total of nine (9) applications. The site was marked to ensure the continuity of patch application. Following supervised removal and scoring of the first Induction patch, participants were instructed to remove all subsequent Induction patches at home, twenty-four hours after application. The evaluation of this site was made again just prior to re-application. If a participant was unable to report for an assigned test day, one (1) makeup day was permitted. This day was added to the Induction period.

With the exception of the first supervised Induction Patch reading, if any test site exhibited a moderate (2-level) reaction during the Induction Phase, application was moved to an adjacent area. Applications were discontinued for the remainder of this test phase, if a moderate (2-level) reaction was observed on this new test site. Applications would also be discontinued if marked (3-level) or severe (4-level) reactivity was noted.

Rest periods consisted of one day following each Tuesday and Thursday removal, and two days following each Saturday removal.

Challenge Phase:

Approximately two (2) weeks after the final Induction patch application, a Challenge patch was applied to a virgin test site adjacent to the original Induction patch site, following the same procedure described for Induction. The patch was removed and the site scored at the clinic Day 1 and Day 3 post-application.

Methodology (continued):

Evaluation Criteria (Erythema and additional Dermal Sequelae):

0	=	**No visible skin reaction**	**E**	=	**Edema**
0.5	=	**Barely perceptible**	**D**	=	**Dryness**
1	=	**Mild**	**S**	=	**Staining**
2	=	**Moderate**	**P**	=	**Papules**
3	=	**Marked**	**V**	=	**Vesicles**
4	=	**Severe**	**B**	=	**Bullae**
			U	=	**Ulceration**
			Sp	=	**Spreading**

Erythema was scored numerically according to this key. If present, additional Dermal Sequelae were indicated by the appropriate letter code and a numerical value for severity.

Adverse Events:

On 4/12/19, Subject #40 had an allergic reaction on his face. His private physician prescribed Methylprednisolone, which he took for four days. On 4/19/19, a follow-up conversation revealed that he was feeling better. The Principal Investigator discontinued his participation for taking a prohibitive medication. He judged the severity of the response as moderate, but unlikely related to the test material.

Amendments:

There were no amendments.

Deviations:

There were no deviations.

Results:

The results of each participant are appended (Table 1).

Observations remained negative throughout the test interval.

Subject demographics are presented in Table 2.

Summary:

Under the conditions of this study, test material, M15019MAX Leucidal® SF Max Lot: 6647, indicated no potential for dermal irritation or allergic contact sensitization.

Table 1
Panel #20190145

Individual Results

M15019MAX Leucidal® SF Max Lot: 6647

Subject Number	Induction Phase									Virgin Challenge Site	
	Day1*	1	2	3	4	5	6	7	8	9	Day 1*	Day 3
1	----------DID NOT COMPLETE STUDY----------											
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	----------DID NOT COMPLETE STUDY----------							
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	---DID NOT COMPLETE STUDY---				
13	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	---DID NOT COMPLETE STUDY---				
17	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	----------DID NOT COMPLETE STUDY----------								
23	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0

Day 1* = Supervised removal

Table 1
(continued)
Panel #20190145

Individual Results

M15019MAX Leucidal® SF Max Lot: 6647

Subject Number	Induction Phase									Virgin Challenge Site	
	Day1*	1	2	3	4	5	6	7	8	9	Day 1* Day 3
30	0	0	0	0	0	0[m]	0	0	0	0	0 0
31	0	0	0	0	0	0	0	0	0	0	0 0
32	0	0	0	0	0	0	0	0	0[m]	0	0 0
33	0	0	0	0	0	0	0	0	0	0	0 0
34	0	0	0	0	0	0	0	0	0	0	0 0
35	0	0	0	0	0	0	0	0	0	0	0 0
36	0	0	0	0	0	0	0	0	0	0	0 0
37	0	0	0	0	0	0	0	0	0	0	0 0
38	0	0	0	0	0	0	0	0	0	0	0 0
39	0	0	0	0	0	0	0	0	0	0	0 0
40	0	0	0	0	0	0	---------DID NOT COMPLETE STUDY---------				
41	0	0	0	0	0	0	0	0	0	0	0 0
42	0	0	0	0	0	0	0	0	0	0	0 0
43	0	0	0	0	0	0	0	0	0	0	0 0
44	0	0	0	0	0	0	0	0	0	0	0 0
45	0	0	0	0	0	0	0	0	0	0	0 0
46	0	0	0	0	0	0	0	0	0	0	0 0
47	0	0	0	0	0	0	0	0	0	0	0 0
48	----------DID NOT COMPLETE STUDY----------										
49	0	0	0	0	0	0	0	0	0	0	0 0
50	0	0	0	0	0	0	0	0	0	0	0 0
51	0	0	0	0	0	0	0	0	0	0	0 0
52	0	0	0	0	0	0	0	0	0	0	0 0
53	0	0	0	0	0	0	0	0	0	0	0 0
54	0	0	0	0	0	0	0	0	0	0	0 0
55	0	0	0	0	0	0	0	0	0	0	0 0
56	0	0	0	0	0	0	0	0	0	0	0 0
57	0	0	0	0	0	0	0	0	0	0	0 0
58	0	0	0	0	0	0	0	0	0	0	0 0

Day 1* = Supervised removal
m = Additional makeup day granted at the discretion of the clinic supervisor

Table 2
Panel #20190145

Subject Demographics

Subject Number	Initials	Age	Gender
1	EVR	72	F
2	EMP	71	F
3	LMF	76	F
4	WEF	77	M
5	M-A	49	F
6	J-K	20	F
7	A-T	49	M
8	SCM	54	F
9	SMA	19	F
10	KSB	61	F
11	KMM	19	F
12	A-C	23	M
13	REM	50	F
14	JRS	29	F
15	N-R	44	F
16	JAM	22	F
17	H-O	73	F
18	KEB	58	M
19	DAG	62	F
20	L-T	58	M
21	VLW	53	F
22	JMC	19	F
23	G-M	20	M
24	KIW	55	F
25	SAT	57	F
26	FTC	59	M
27	B-C	57	F
28	R-O	64	F
29	G-S	44	F

Table 2
(continued)
Panel #20190145

Subject Demographics

Subject Number	Initials	Age	Gender
30	J-R	39	M
31	JRM	27	M
32	A-G	27	M
33	IVL	57	F
34	L-S	45	F
35	P-E	36	M
36	DML	53	F
37	Y-L	29	F
38	SMC	27	F
39	EAD	30	M
40	O-F	54	M
41	G-S	50	F
42	R-M	49	F
43	Z-O	65	M
44	A-B	46	F
45	ASE	51	M
46	F-G	51	M
47	R-G	17	F
48	MJA	47	M
49	CEO	49	F
50	LSR	33	F
51	ABC	42	M
52	GDS	50	F
53	DNS	16	F
54	JEY	48	F
55	E-G	46	M
56	LPR	28	F
57	D-R	30	M
58	YRG	42	M

Bacterial Reverse Mutation Test

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(704) 276-7100 • Fax (704) 276-7101

Test Article: Leucidal® SF Max 100% Lactobacillus Ferment
Code Number: M15019MAX
CAS #: 1686112-36-6 (or) 68333-16-4

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

Study Director: *Maureen Danaher*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part3

Test Request Number: 4427

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® SF Max** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C 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 37^0C. 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 WP2*uvr*A. 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

ACTIVE MICRO TECHNOLOGIES

Bacterial Reverse Mutation Test

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(704) 276-7100 • Fax (704) 276-7101

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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* WP2uvrA 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.

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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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

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* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml. The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

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 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

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C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. 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 WP2*uvr*A 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.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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* WP2*uvr*A.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.

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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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Bacterial Reverse Mutation Test

	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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Bacterial Reverse Mutation Test

	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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*WP2uvrA*		
		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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Dermal and Ocular Irritation Tests

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Tradename: Leucidal® SF Max 100% Lactobacillus Ferment

Code: M15019MAX

CAS #: 1686112-36-6 (or) 68333-16-4

Test Request Form #: 3686

Lot #: NC170915-E

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

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® SF Max** 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% CO_2, 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.

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Dermal and Ocular Irritation Tests

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II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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% CO_2 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% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) 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.

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 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.

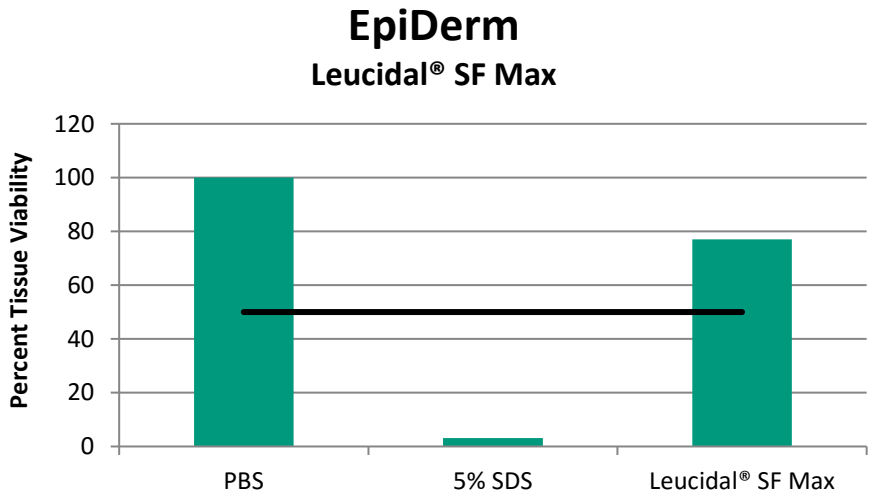

Figure 1: EpiDerm tissue viability

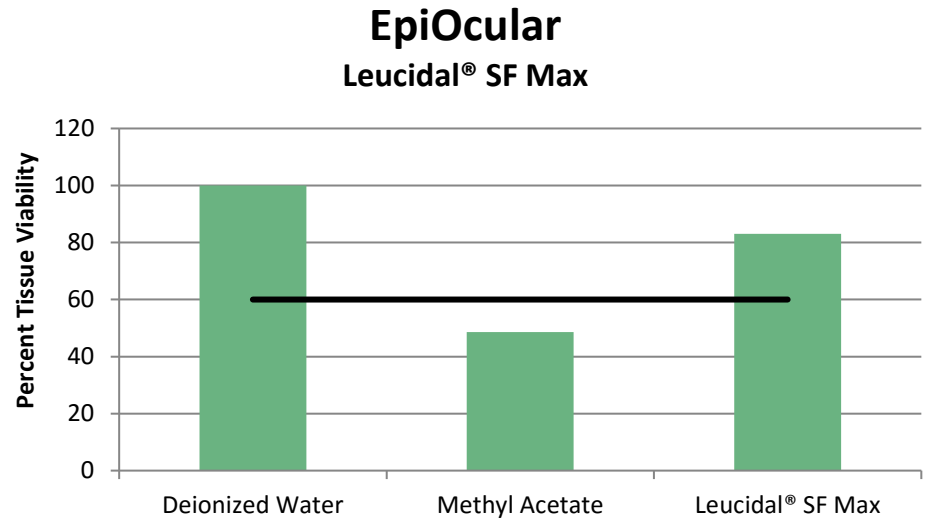

Figure 2: EpiOcular tissue viability

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: April 25, 2025

SUBJECT: Lactobacillus Ferment Lysate Filtrate

Active Concepts LLC. 2017. Product Specification ACB Yogurt Dermal Respiratory Factor.

AMA Laboratories Inc. 2008. 50 Human Subject Repeat Insult Patch Test Skin Irritation/Sensitization Evaluation (occlusive patch) ACB Yogurt Dermal Respiratory Factor (Lactobacillus Ferment Lysate Filtrate) (test material was a product containing 5% Lactobacillus Ferment Lysate Filtrate).

Active Concepts LLC. 2016. Dermal and Ocular Irritation Tests ACB Phyto-Biotics Bifidus (Lactobacillus Ferment Lysate Filtrate).

Active Concepts LLC. 2016. Bacterial Reverse Mutation Assay ACB Phyto-Biotics Bifidus (Lactobacillus Ferment Lysate Filtrate).

Product Specification

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

made from Lactobacillus bulgaricus
suggested use level: 2-5%
99.1% Lactobacillus Ferment Lysate Filtrate (10-16% solids)

Product Name: ACB Yogurt Dermal Respiratory Factor
Code Number: 20224
CAS #'s: 68333-15-3
EINECS #'s: N/A
INCI Name: Lactobacillus Ferment Lysate Filtrate
Status: Approved

Specification	Parameter
Appearance	Clear to Slightly Hazy Semi-Viscous Liquid
Color	5 Gardner Maximum
Odor	Characteristic
pH (Direct)	3.5 – 5.5
Solids (1g-1hr-105^{0}C)	10.0 – 16.0%
Specific Gravity (25^{0}C)	1.020 – 1.065
Heavy Metals Lead Arsenic Cadmium	< 20 ppm < 10 ppm < 2 ppm < 1 ppm
Microbial Content Aerobic mesophiles Anaerobic mesophiles Mold and yeast Pathogens	 < 100 CFU/g < 10 CFU/g < 100 CFU/g None present

May Sediment upon Standing; Mix Well Prior to Use

**Note: Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored.

AMA LABORATORIES, INC.
216 Congers Road, Bldg. 1
New City, NY 10956 USA
(845) 634-4330
FAX: (845) 634-5565
www.amalabs.com

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

ACB Yogurt Dermal Respiratory Factor (Lactobacillus Ferment Lysate Filtrate)

AMA Ref. No.: MS08.RIPT.L2089O.50.ACTC

Date: February 18, 2008

Sponsor: Active Concepts, LLC
121 Ethel Road West, Suite 3
Piscataway, New Jersey 08854

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:

Test material was a product containing 5% Ferment Lysate Filtrater

2.1 Test Material Description:

On January 11, 2008 one test sample labeled EN080110-C was received from Active Concepts, LLC and assigned AMA Lab No. L-2089.

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		26-64
Sex	Male	7
	Female	45
Race	Caucasian	42
	Hispanic	9
	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.
- 0.2 ml or 0.2g 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 Conclusions:

The test material (AMA Lab. No.: L-2089; Client No.: EN080110-C) when tested under occlusion 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

Patrycja Bienias, M.S.
Technician

David R. Winne, B.S.
Technical Director

2/18/08
Date

TABLE
SUMMARY OF RESULTS
(Occlusive Patch)

AMA Lab No.: L-2089
Client No.: EN080110-C

No.	Subject ID	RACE	SEX	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	28 0971	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
3	34 4672	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
4	36 2168	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
5	36 7304	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
6	36 7970	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
7	36 8248	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
8	40 6489	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
9	42 1835	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
10	42 1837	C	F	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
11	44 9258	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
12	46 4172	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
13	48 4004	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
14	50 1699	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
15	50 1729	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
16	50 3800	A	M	0	0	0	0	0	0	0	0	0	0	0	0.0
17	50 5772	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
18	50 8253	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
19	52 4898	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
20	52 5000	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
21	54 0763	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
22	54 1935	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
23	54 2951	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
24	54 4408	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
25	54 6357	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
26	56 0719	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
27	56 3659	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
28	56 4962	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
29	56 5529	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.: L-2089
Client No.: EN080110-C

No.	Subject ID	RACE	SEX	Response									Chall.		Score
				1	2	3	4	5	6	7	8	9	24 HR	48 HR	
30	58 3087	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
31	58 3965	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
32	58 7412	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
33	58 9750	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
34	60 0082	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
35	60 1825	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
36	60 2888	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
37	60 3135	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
38	60 6328	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
39	60 9336	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
40	62 3596	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
41	62 5624	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
42	62 8070	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
43	64 2464	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
44	64 4340	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
45	64 6653	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
46	64 8003	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
47	66 1927	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
48	70 5391	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
49	72 2318	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
50	76 2719	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
51	82 4417	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
52	90 3845	H	F	0	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A

Evaluation Period:

This study was conducted from January 14, 2008 through February 15, 2008.

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.

13.0 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:

Kamil Wojtowicz

Kamil Wojtowicz, M.S.
Quality Assurance Supervisor

2/18/08
Date

Dermal and Ocular Irritation Tests

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Trade Name: ACB Phyto-Biotics Bifidus

98% Lactobacillus Ferment Lysate Filtrate
solids 24% minumum (usually about 25%)

made from Lactobacillus bifidus
suggested use level: 1-10%

Code: 16600

CAS #: N/A

Test Request Form #: 806

Lot #: SN130909-5

Sponsor: *Active Concepts, LLC; 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 **ACB Phyto-Biotics Bifidus** 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% CO_2, 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.

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Dermal and Ocular Irritation Tests

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.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 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 H_2O
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%.

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Dermal and Ocular Irritation Tests

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 Concepts, 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% CO_2 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% CO_2 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% CO_2, 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% CO_2, 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% CO_2, 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 (OD_{570}) 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%.

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Dermal and Ocular Irritation Tests

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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 ≤ 50% for EpiDerm™ or ≤ 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.

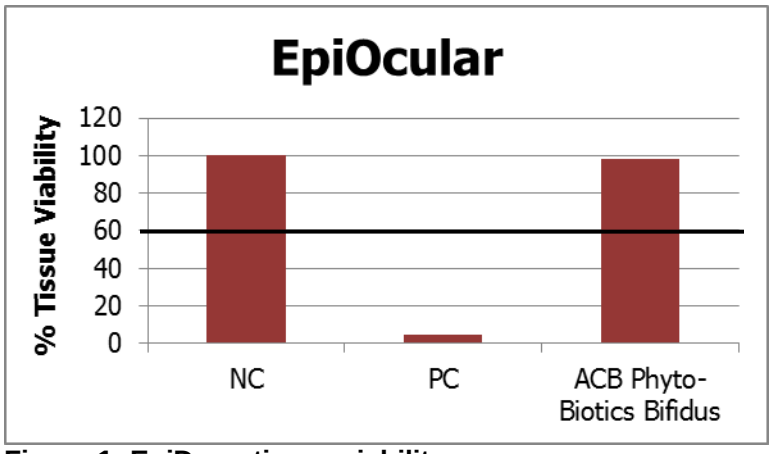

Figure 1: EpiDerm tissue viability

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Dermal and Ocular Irritation Tests

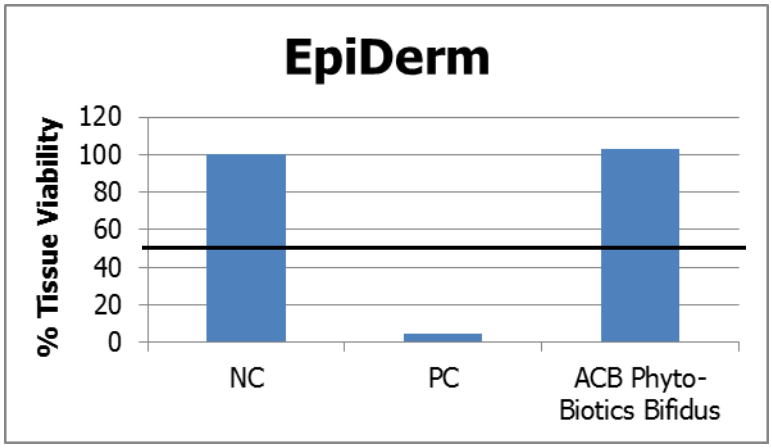

Figure 2: EpiOcular tissue viability

Bacterial Reverse Mutation Test

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Test Article: ACB Phyto-Biotics Bifidus
Code Number: 16600
CAS #: N/A

Lactobacillus Ferment Lysate Filtrate

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

Study Director: *Maureen Danaher*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 2593

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 **ACB Phyto-Biotics Bifidus** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A 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 WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^{0}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^{0}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 WP2*uvr*A. 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.

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Bacterial Reverse Mutation Test

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* WP2*uvr*A 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.

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 *uvr*B gene and the deletion in the *uvr*A 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*uvr*A) 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.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	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.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA 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 reveratants 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 $2x10^9$/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 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl 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 50µl 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 reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants 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* and *Escherichia coli* 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.

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B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. 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 WP2*uvr*A 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 mete 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* WP2*uvr*A.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.

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Bacterial Reverse Mutation Test

Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	***TA98***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	37	37	37
	1500	25	32	29
	500	28	21	25
	150	32	32	32
	50	30	31	31
	15	27	42	35
	5.0	22	44	33
	1.5	18	18	18
Test Solution w/o S9	5000	25	41	33
	1500	25	21	23
	500	33	22	28
	150	28	25	27
	50	47	41	44
	15	36	26	31
	5.0	25	17	21
	1.5	48	18	33
DI Water w/S9		55	46	51
DI Water w/o S9		58	42	50
2-aminoanthracen w/ S9		236	235	236
2-nitrofluorene w/o S9		246	217	232
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	216	223	220
	1500	210	187	199
	500	140	125	133
	150	117	127	122
	50	148	121	135
	15	123	116	120
	5.0	126	147	137
	1.5	132	123	128
Test Solution w/o S9	5000	118	145	132
	1500	98	95	97
	500	101	135	118
	150	112	125	119
	50	178	163	171
	15	145	136	141
	5.0	115	138	127
	1.5	110	102	106
DI Water w/S9		236	225	231
DI Water w/o S9		230	245	238
2-aminoanthracen w/ S9		585	566	576
Sodium azide w/o S9		635	688	662
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*TA1537*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	18	25	22
	1500	21	18	20
	500	20	21	21
	150	33	33	33
	50	17	20	19
	15	25	28	27
	5.0	25	20	23
	1.5	25	22	24
Test Solution w/o S9	5000	21	32	27
	1500	16	28	22
	500	17	22	20
	150	25	28	27
	50	23	24	24
	15	21	36	29
	5.0	21	21	21
	1.5	21	23	22
DI Water w/S9		49	59	54
DI Water w/o S9		65	63	64
2-aminoanthracen w/ S9		411	436	424
2-aminoacridine w/o S9		345	366	356
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	22	25	24
	1500	29	31	30
	500	27	27	27
	150	23	35	29
	50	36	31	34
	15	25	24	25
	5.0	28	26	27
	1.5	20	26	23
Test Solution w/o S9	5000	31	30	31
	1500	20	20	20
	500	28	30	29
	150	32	45	39
	50	24	25	25
	15	15	22	19
	5.0	36	19	28
	1.5	17	13	15
DI Water w/S9		55	59	57
DI Water w/o S9		46	52	49
2-aminoanthracen w/ S9		266	256	261
Sodium azide w/o S9		621	601	611
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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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	35	28	32
	1500	18	38	28
	500	33	25	29
	150	23	18	21
	50	20	23	22
	15	21	35	28
	5.0	25	15	20
	1.5	23	22	23
Test Solution w/o S9	5000	28	33	31
	1500	18	16	17
	500	22	16	19
	150	25	20	23
	50	32	31	32
	15	23	20	22
	5.0	21	23	22
	1.5	28	27	28
DI Water w/S9		50	63	57
DI Water w/o S9		66	78	72
2-aminoanthracen w/ S9		266	250	258
Methylmethanesulfonate w/o S9		278	211	245
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

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: April 28, 2025

SUBJECT: Lactobacillus Ferment Ingredients

Anonymous. 2025. Summary Composition Information of Lactobacillus Ferment Ingredients Derived-From Various Lactobacillus Species.

Anonymous. 2025. Manufacturing Flow Chart A (This chart applies to ingredients numbered 1, 2, 4, 5, 8, 9, 11, 12, 13,14, 16, 19, 20, 21, 22, 26, and 28).

Anonymous. 2025. Manufacturing Flow Chart B (This chart applies to ingredients numbered 3, 6, 10, 17, 25 and 29).

Anonymous. 2025. Manufacturing Flow Chart C (This chart applies to the ingredient numbered 7).

Anonymous. 2025. Manufacturing Flow Chart D (This chart applies to ingredient numbered 15).

Anonymous. 2025. Manufacturing Flow Chart E (This chart applies to ingredient numbered 18).

Anonymous. 2025. Manufacturing Flow Chart F (This chart applies to ingredients numbered 23 & 24).

April 2025

Summary Composition Information of Lactobacillus Ferment Ingredients Derived-From Various Lactobacillus Species

Product No.	Scientific Name	INCI Name	Composition	Manufacturing Flow Chart
1	*Lactobacillus curvatus*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
2	*Lactobacillus plantarum*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
3	*Lactobacillus plantarum*	Lactobacillus Ferment Filtrate	98% Lactobacillus Ferment Filtrate 2% 1,2-Hexanediol	B
4	*Lactobacillus plantarum*	Lactobacillus Ferment	87.9% Lactobacillus Ferment 10% Butylene Glycol 2% 1,2-Hexanediol 0.1% Ethylhexylglycerin	A
5	*Lactobacillus brevis*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
6	*Lactobacillus brevis*	Lactobacillus Ferment Lysate	98% Lactobacillus Ferment 2% 1,2-Hexanediol	B
7	*Lactobacillus plantarum*	Lactobacillus Ferment Lysate	98% Lactobacillus Ferment 2% 1,2-Hexanediol	C
8	*Lactobacillus plantarum*	Lactobacillus Ferment	97% Lactobacillus Ferment 3% 1,2-Hexanediol	A
9	*Lactobacillus rhamnosus*	Lactobacillus Ferment	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	A
10	*Lactobacillus rhamnosus*	Lactobacillus Ferment Filtrate	97.8% Lactobacillus Ferment 2.2% 1,2-Hexanediol	B
11	*Lactobacillus brevis*	Lactobacillus Ferment	97.8% Lactobacillus Ferment 2.2% 1,2-Hexanediol	A
12	*Lactobacillus pentosus*	Lactobacillus Ferment	98% Lactobacillus Ferment	A

			2% 1,2-Hexanediol	
13	*Lactobacillus plantarum*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
14	*Lactobacillus plantarum*	Lactobacillus Ferment	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	A
15	*Lactobacillus plantarum*	Lactobacillus Ferment	0.1% Lactobacillus Ferment 69.9% Water 30% Butylene Glycol	D
16	*Lactobacillus brevis*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
17	*Lactobacillus kunkeei*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	B
18	*Lactobacillus kunkeei*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	E
19	*Lactobacillus plantarum*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
20	*Lactobacillus paracasei*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
21	*Lactobacillus paracasei*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
22	*Lactobacillus paracasei*	Lactobacillus Ferment	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	A
23	*Lactobacillus brevis*	Lactobacillus Ferment Lysate	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	F
24	*Lactobacillus brevis*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	F
25	*Lactobacillus plantarum*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	B
26	*Lactobacillus reuteri*	Lactobacillus Ferment	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	A
27	*Lactobacillus plantarum*	Lactobacillus Ferment	99% Lactobacillus Ferment	B

			0.8% Propanediol 0.15% Caprylyl Glycol 0.05% Ethylhexylglycerin	
28	*Lactobacillus rhamnosus*	Lactobacillus Ferment	98% Lactobacillus Ferment 2% 1,2-Hexanediol	A
29	*Lactobacillus reuteri*	Lactobacillus Ferment Filtrate	97.5% Lactobacillus Ferment 2.5% 1,2-Hexanediol	B

April 2025

Manufacturing Flow Chart A

Lactobacillus Ferment ingredients as listed in the summary table

This chart applies to ingredients numbered 1, 2, 4, 5, 8, 9, 11, 12, 13,14, 16, 19, 20, 21, 22, 26, and 28.

Media Preparation

↓

Sterilization & Filtration

↓

Fermentation (Add *Lactobacillus* species specified in the summary tables)

↓

Sterilization & Filtration

↓

Decolorization & Deodorization

↓

Addition (other ingredients as listed in summary tables, e.g., 1,2-Hexanediol)

↓

Stabilization

↓

Quality Control

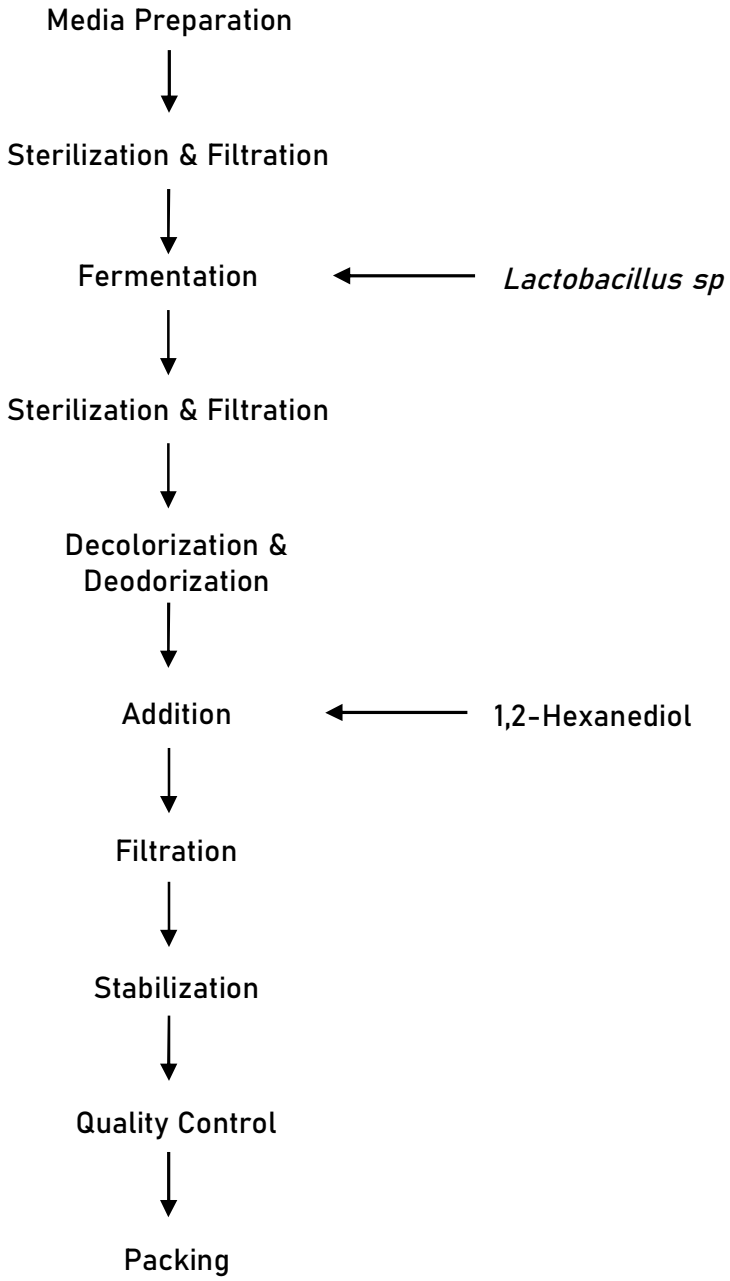
↓

Packing

Manufacturing Flow Chart B

Applies to numbers 3, 6, 10, 17, 25 and 29 in the summary table

LACTOBACILLUS FERMENT FILTRATE (derived from *Lactobacillus sp*)



Manufacturing Flow Chart C

LACTOBACILLUS FERMENT LYSATE (derived from *Lactobacillus plantarum*)

Media Preparation

- Glucose
- Yeast Extract
- Soy peptone

Media Preparation

↓

Sterilization

↓

Fermentation ← *Lactobacillus plantarum*

35℃, 72hr

↓

Quality Control

↓

Tyndallization

80℃, 1hr/day, 3days

↓

Addition ← 1,2-Hexanediol

↓

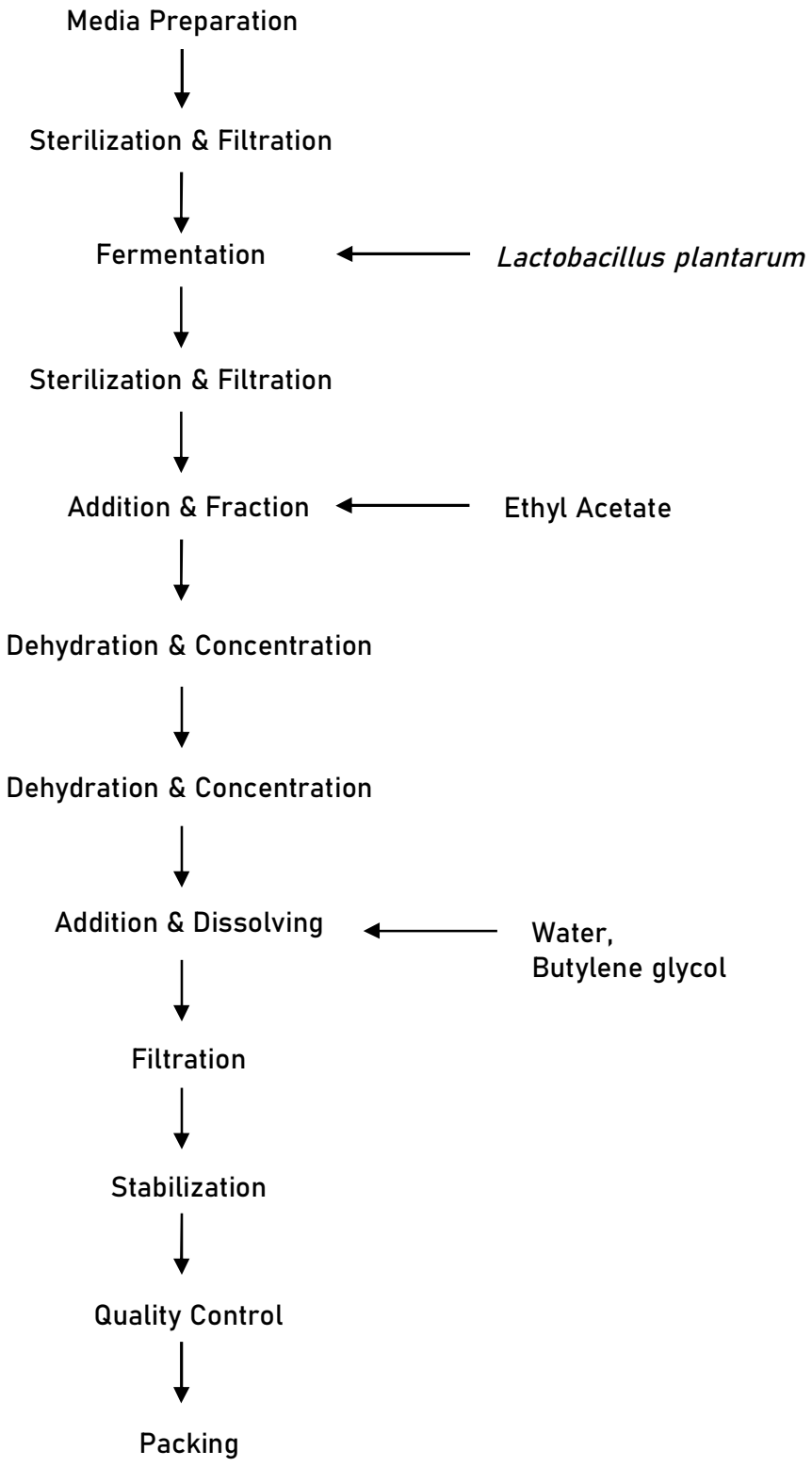
Quality Control

↓

Packing

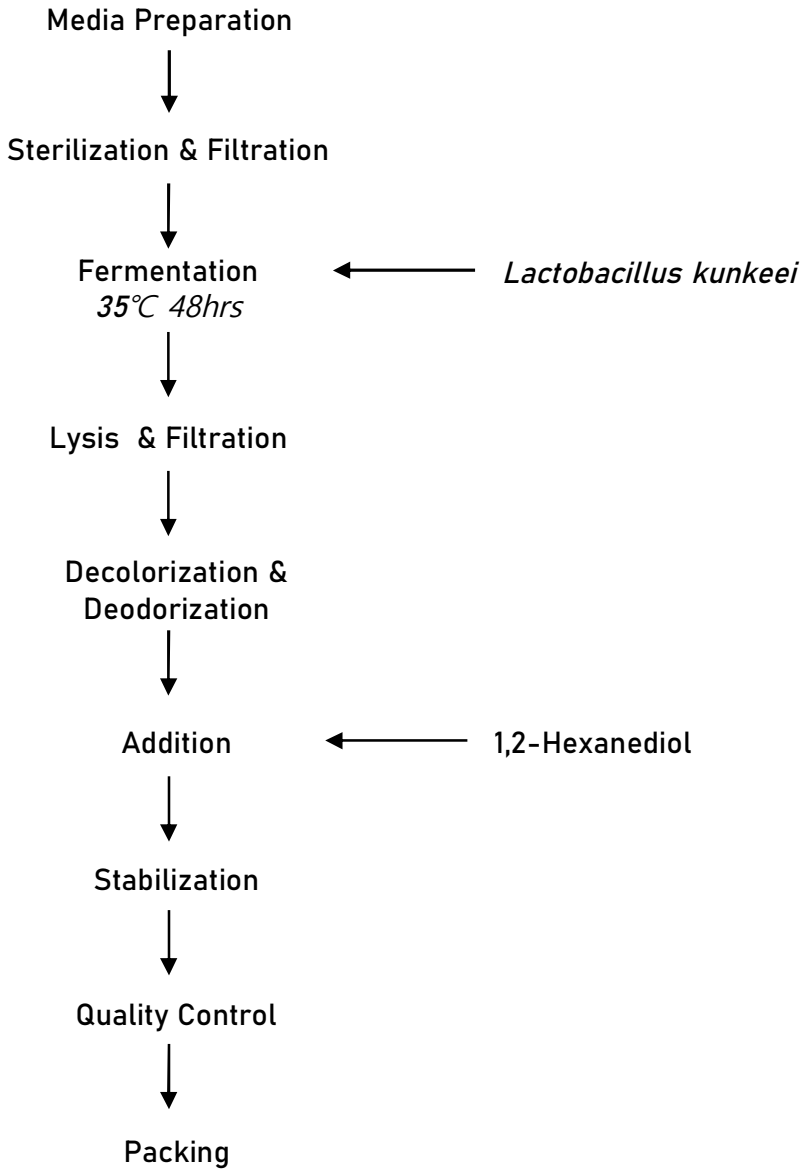
Manufacturing Flow Chart D

LACTOBACILLUS FERMENT (derived from *Lactobacillus plantarum*)



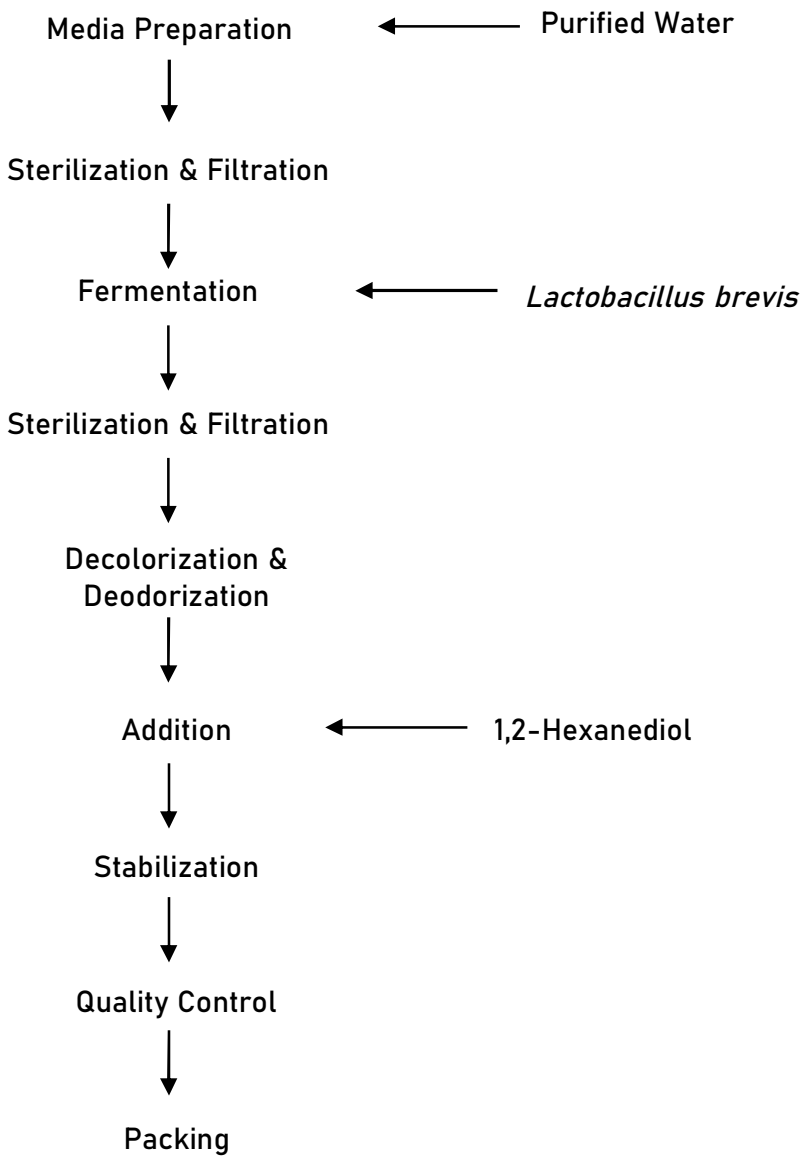
Manufacturing Flow Chart E

LACTOBACILLUS FERMENT (derived from *Lactobacillus kunkeei*)



Manufacturing Flow Chart F

LACTOBACILLUS FERMENT (derived from *Lactobacillus brevis*)



Concentration of Use by FDA Product Category – Lactobacillus Ferments*

Lactobacillus Ferment
Lactobacillus Ferment Lysate
Lactobacillus Ferment Lysate Filtrate
Lactobacillus Ferment Filtrate

Ingredient	Product Category	Maximum Concentration of Use
Lactobacillus Ferment	Eye lotions	0.1%
Lactobacillus Ferment	Other hair preparations (noncoloring)	0.001%
Lactobacillus Ferment	Makeup bases	1.5%
Lactobacillus Ferment	Other makeup preparations	0.19%
Lactobacillus Ferment	Skin cleansing (cold creams, cleansing lotions, liquids, and pads)	0.04%
Lactobacillus Ferment	Face and neck products Not spray	0.18%
Lactobacillus Ferment	Moisturizing products Not spray	0.01%
Lactobacillus Ferment	Skin fresheners	0.2%
Lactobacillus Ferment Lysate	Eye lotions	0.5%
Lactobacillus Ferment Lysate	Paste masks and mud packs	0.17%
Lactobacillus Ferment Filtrate	Skin cleansing (cold creams, cleansing lotions, liquids, and pads)	0.0005%
Lactobacillus Ferment Filtrate	Face and neck products Lotion not spray	0.09%

*Ingredients included in the title of the table but not found in the table were included in the concentration of use survey, but no uses were reported

Information collected in 2022
Table prepared: July 6, 2022