## ACB Bio-Chelate 5 PF Efficacy Data

<table>
<thead>
<tr>
<th>Code:</th>
<th>20339PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>INCI Name:</td>
<td>Water &amp; Saccharomyces/Zinc Ferment &amp; Saccharomyces/Copper Ferment &amp; Saccharomyces/Magnesium Ferment &amp; Saccharomyces/Iron Ferment &amp; Saccharomyces/Silicon Ferment</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Study</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair Smoothing</td>
<td>Analysis of the treated fibers by SEM demonstrated that <strong>ACB Bio-Chelate 5 PF</strong> smoothed of the hair cuticle, without surface buildup of the treatment product.</td>
</tr>
<tr>
<td>High Resolution Skin-Imaging Assay</td>
<td><strong>ACB Bio-Chelate 5 PF</strong> improved skin density by 11.02%, after 24 hours and by 13.54% after 4 weeks when compared to the untreated control. When compared to the base cream <strong>ACB Bio-Chelate 5 PF</strong> improved skin density by 14.48% after 24 hours and after 4 weeks <strong>ACB Bio-Chelate 5 PF</strong> improved density.</td>
</tr>
<tr>
<td>ORAC Assay</td>
<td>Oxygen Radical Absorbance Capacity (ORAC) is a measure of a material's potential to protect against oxidative stress or reactive oxygen species (ROS). <strong>ACB Bio-Chelate 5 PF</strong> demonstrated significant antioxidant activity by reducing the presence of ROS compared with Trolox®, the vitamin E analog.</td>
</tr>
<tr>
<td>Gene Expression</td>
<td>Results illustrated that 1,155 genes were altered by 30%. 591 genes were down regulated and 564 were up regulated. It is clear that bio-available minerals have an impact when applied topically. The cosmetic industry’s claims that minerals can relax the skin and have anti-inflammatory properties are supported by this data.</td>
</tr>
</tbody>
</table>
Cellular Viability Assay

In this study, ACB Bio-Chelate 5 PF (code 20339PF) was tested to evaluate its effects on the viability of normal human dermal fibroblasts (NDHF). At concentrations of both 0.1% and 0.01% ACB Bio-Chelate 5 PF (code 20339PF), nor the preservatives contained therein exhibited any inhibition of cell viability. It can therefore be concluded that at normal use concentrations ACB Bio-Chelate 5 PF (code 20339PF) is not cytotoxic.
Objective

To determine the ability of ACB Bio-Chelate 5 PF to smooth the cuticle of the hair as well as its capability to penetrate into the hair shaft. Energy Dispersive Analysis of X-rays as well as Scanning Electron Microscopy were used to evaluate the results of the studies. According to the results, ACB Bio-Chelate 5 PF is capable of significantly smoothing the hair cuticle for healthier and shiner looking hair while simultaneously nourishing it as it is capable of enhancing the delivery of minerals into the shaft.

Materials and Methods

Hair efficacy was performed employing ACB Bio-Chelate 5 PF, a mineral glycopeptide complex containing Mg, Fe, Zn, Cu, and Si. Scanning Electron Microscopy (SEM) was utilized to observe the surface architecture of the hair strands. Energy Dispersive Analysis of X-Rays (EDAX) was used to determine the elemental composition of the hair below the surface.

Results

Figure 1: Elemental Composition by EDAX analysis of mineral in the hair

Figure 2: Scan Electron Microscopy of hair shaft following application of ACB Bio-Chelate 5 PF
Discussion

EDAX studies confirmed penetration of magnesium and silicon. The EDAX scan (Figure 1) shows the untreated controls in red and the ACB Bio-Chelate 5 PF treated samples as a black superimposed line. European blonde hair tresses were damaged by a 2x-bleach/wave process followed by a 30-50% elongation of the individual hair fibers in order to stimulate excessive chemical and physical damage. Three fibers were cut into equal halves to produce six hair strands. Three hair strands functioned as the test materials, while the remaining three functioned as controls. The three damaged test fibers were treated with 100% ACB Bio-Chelate 5 and allowed to dry. Analysis of the treated fibers by SEM (Figure 2) demonstrated a smoothing of the hair cuticle, without a surface buildup of the treatment product.
Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the effect on skin density of **ACB Bio-Chelate 5 PF**. 10 M/F subjects between the ages of 23-45 participated in the study. Data gathered from the high resolution ultrasound imaging yielded results that indicate that this material is capable of significantly improving skin density compared to the control.

Materials

A. Equipment: DermaLab Skin Combo (Ultrasound Probe)

Methods

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

The intensity of the signals that are received refer to a color scale. Dark colors represent areas of the skin with low reflection. This means that there are no changes or very small changes in density between the structures in the skin. Bright colors represent areas with strong reflections, signifying substantial changes in density between structures.
10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. The DermaLab ultrasound probe was used to determine the skin density of the subject's volar forearms.

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken 24 hours after application of test materials and then weekly for 4 weeks. The test material consisted of 2.0% **ACB Bio-Chelate 5 PF** in a base lotion.

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

**Results**

**ACB Bio-Chelate 5 PF** showed improvements in skin density at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

**Raw Data:**

<table>
<thead>
<tr>
<th></th>
<th>t = 24</th>
<th>1 week</th>
<th>2 week</th>
<th>3 week</th>
<th>4 week</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental (2.0% ACB Bio-Chelate 5 PF in Base Lotion)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>54.4</td>
<td>59.0</td>
<td>60.9</td>
<td>58.9</td>
<td>66.7</td>
</tr>
<tr>
<td>Untreated</td>
<td>58.6</td>
<td>60.9</td>
<td>64.1</td>
<td>67.1</td>
<td>69.1</td>
</tr>
<tr>
<td>Base Lotion Control</td>
<td>52.1</td>
<td>62.4</td>
<td>68.1</td>
<td>61.0</td>
<td>69.2</td>
</tr>
<tr>
<td><strong>Experimental vs. Untreated</strong></td>
<td>11.02%</td>
<td>13.08%</td>
<td>15.07%</td>
<td>12.25%</td>
<td>13.54%</td>
</tr>
<tr>
<td><strong>Base Lotion vs. Untreated</strong></td>
<td>1.01%</td>
<td>2.46%</td>
<td>3.21%</td>
<td>2.25%</td>
<td>1.16%</td>
</tr>
<tr>
<td><strong>Experimental vs. Base Lotion</strong></td>
<td>14.48%</td>
<td>15.41%</td>
<td>13.26%</td>
<td>13.46%</td>
<td>13.69%</td>
</tr>
</tbody>
</table>
High Resolution Ultrasound Skin Imaging Assay

Discussion

As evidenced in a 4 week efficacy study of **ACB Bio-Chelate 5 PF** on skin, skin density was improved by 11.02% after 24 hours and by 13.54% after 4 weeks when compared to the untreated control. When compared to the base cream **ACB Bio-Chelate 5 PF** improved skin density by 14.48% after 24 hours and after 4 weeks **ACB Bio-Chelate 5 PF** improved density by 13.69%. Results indicate that **ACB Bio-Chelate 5 PF** is capable of improving skin density when compared to both the untreated control as well as the base lotion.

**ACB Bio-Chelate 5 PF** has a positive effect on skin’s density when used at recommended use levels.
**Tradename:** ACB Bio-Chelate 5 PF  

**Code:** 20339PF


**Test Request Form #:** 201

**Lot #:** 26738

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092  
**Study Director:** Erica Segura  
**Principle Investigator:** Meghan Darley

**Test Performed:** 
Oxygen Radical Absorbance Capacity (ORAC)

**Introduction**

Reactive oxygen species (ROS) are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS are detrimental to cellular structures and functional molecules (i.e. DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. The oxygen radical absorbance capacity (ORAC) assay is a standard method used to assess antioxidant capacity of physiological fluids, foods, beverages, and natural products. The assay quantitatively measures a sample’s ability to quench free radicals that have the potential to react with and damage cellular components.

Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of ACB Bio-Chelate 5 PF.

**Assay Principle**

This assay is based upon the effect of peroxyl radicals generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The degree of change is indicative of the amount of radical damage and the presence of antioxidants results in an inhibition in the free radical damage to the fluorescein. The antioxidant protection of the sample can be calculated by comparing it to a set of known standards. Trolox®, a water soluble vitamin E analog, with known antioxidant capabilities is used in this ORAC assay as the standard for measuring the antioxidant capacity of unknown substances. ORAC values, expressed in µM of Trolox® equivalents (TE), are calculated using the area under the curves (AUC) of the test product, Trolox®, and the control materials. Trolox equivalency is used as the benchmark for antioxidant capacity of mixtures since it is difficult to measure individual components.
Oxygen Radical Absorbance Capacity (ORAC) Assay

Materials

A. Equipment: Synergy H1 Microplate reader (BioTek Instruments, Winooski, VT); Gen5 software (BioTek Instruments, Winooski, VT); Pipettes
B. Buffers: 75mM Potassium Phosphate (pH 7.4); Deionized H₂O
C. Reagents: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)
D. Preparation: Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.
E. Microtitre Plates: Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of ACB Bio-Chelate 5 PF and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared at a concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37°C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

\[
\text{AUC} = 0.5 + \frac{R_2}{R_1} + \frac{R_3}{R_1} + \frac{R_4}{R_1} + \cdots + \frac{R_n}{R_1} \rightarrow \text{Where } R \text{ is fluorescence reading}
\]

\[
\text{Net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}
\]

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample’s Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents (µMTE), where 1 ORAC unit is equal to 1 µMTE.
Results

ACB Bio-Chelate 5 PF began exhibiting antioxidant activity at a 0.5% concentration.

![Figure 1: Antioxidant capacities](image)

Discussion

As shown in figure 1, ACB Bio-Chelate 5 PF exhibited antioxidant activity comparable to 100µM Trolox®. The antioxidant capacity of ACB Bio-Chelate 5 PF increased as the concentration increased, as a result we can assure that its ability to minimize oxidative stress is dose dependent.

ACB Bio-Chelate 5 PF was designed to have conditioning and nourishing properties for skin and hair applications. With the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing potent antioxidant benefits when added to cosmetic applications.
Gene Expression Profile of a Tissue Model Treated with Five Bio-Chelated Minerals

Introduction

Minerals play an essential role in enzymatic reactions and biological systems. The elements important for cellular function include iron, copper, zinc, magnesium, and silicon. Considered trace nutrients, they are often found in commercial supplements. Over the past several years, their use in cosmetics and cosmetic advertising has grown dramatically. At first, minerals like copper and titanium dioxide were added to provide color. They enjoy the benefit of being simple, natural components consumers feel comfortable with. As the population at large learned more about the role of nutrients in their diets, minerals with biological activity were added to capitalize on the available research and health claims. This study investigates the biological activity of bio-chelated minerals topically applied to a differentiated, full-thickness tissue model: A DNA microarray was used to uncover skin’s genetic response to a mineral blend processed to maximize bio-availability.

Experimental

Minerals are available in a variety of forms. One of the most efficient and effective is called bio-chelated or yeast [1]. In this form, yeast is grown in a nutrient medium enriched with inorganic salts. Over several generations, the yeast culture absorbs and processes the added minerals. The resulting yeast extract contains natural combinations of organic compounds and minerals: bio-chelates. In this study, a solution of iron, copper, zinc, magnesium, and silicon chelates was applied to a MatTek tissue model consisting of both keratinocytes and fibroblasts. These models have been shown to have similar gene expression profiles to normal human skin [2].

To learn about the biological activity of topically applied minerals, we employed a DNA microarray with 21,629 different DNA fragments. Given the wide range of health claims made on behalf of mineral supplements, this is an excellent method for comprehensively exploring possible effects. It provides a starting point for subsequent studies designed to support specific claims.

In short, cells regulate gene expression to control their structure and function. Cells express one set of genes to respond to radiation damage and another set to reproduce. This expression results in mRNA copies of the activated genes, or fewer copies in the case of reduced expression. mRNA is the biological template for creating proteins and is the cellular component collected from the tissue models in this test. Treated tissue will have different levels of some mRNA when compared to an untreated control.
Results

Given the established biological activity of the five minerals and the associated yeast extract, it is no surprise that 1,155 genes were altered by more than the 30% limit chosen for this study. 591 were down-regulated, and 564 were up-regulated by a twenty-four hour exposure to a 5% solution of bio-chelated minerals. In contrast, several hundred genes were found to be altered in a study of intrinsic aging [3]. Most of the affected genes have no known role in human skin. They may have been identified in other tissues, species, or not at all. The dearth of available information is noteworthy.

That said, the goal of this research project is to verify the biological activity of topically-applied, bio-chelated mineral blends and identify possible benefits. To analyze the data, the results were first run through the internet software program DAVID. DAVID is an acronym for Database for Annotation, Visualization, and Integrated Discovery. The software identified five KEGG pathways as being particularly affected. KEGG, an acronym for Kyoto Encyclopedia of Genes and Genomes, is a database collection of diagrams representing molecular interaction networks. The five highlighted networks are:

1) Tight Junction: 8 genes up-regulated
2) Cell Adhesion Molecules (CAMS): 8 genes up-regulated
3) Glycerophospholipid Metabolism: 6 genes up-regulated
4) Neuroactive Ligand-Receptor Interaction: 22 genes down-regulated
5) MAPK Signaling Pathway: 15 genes down-regulated

The first three pathways, all up-regulated, affect the skin’s structure and barrier function. Tight junctions seal neighboring cells together in an epithelial sheet to prevent leakage of water-soluble molecules. Cell adhesion molecules function in development, host defense, and tissue organization and repair. They form physical linkages between the extracellular environment and the internal structures to control shape and motility. Glycerophospholipids serve as structural components of cell membranes and are the precursors of lipid mediators involved in signal transduction processes. The interplay between the enzymes, lipids, and their metabolites play an important role in the initiation and maintenance of oxidative stress [4]. The neuroactive ligand and receptor pathway represents 4.1% of the down-regulated list: Keratinocytes and fibroblasts produce neuropeptides and their related receptors as part of a localized stress response system akin to the body’s Hypothalamic-Pituitary-Adrenal Axis [5]. They trigger secondary messenger systems and many result in inflammation. The MAPK signaling pathways react to external stimuli and terminate in ERK, P38, or JNK/SAPK [6]. The P38 and JNK pathways are tied to inflammation.

The DAVID software offers several other ways to study the affected genes via the Functional Annotation Tool. For example, the Gene Ontology Consortium (GO) developed a controlled vocabulary for distributing genes into functional categories. Viewing the Biological Process level, which focuses on broad goals with the least specificity, gives an impression of what the bio-chelated minerals are doing. Table 1 has the top ten statistically-significant GO terms from both the up- and down-regulated lists.
Table 2 has a list of terms representing the greatest number of affected genes with a p-Value of better than 0.05. The p-value, of probability value, is the probability of getting a result as extreme by chance alone. The smaller the p-value, the better the odds that the result is significant.

**Table 1**: Top ten most statistically significant GO terms for the affected genes in both the up- and down regulated lists.

<table>
<thead>
<tr>
<th>GO Term: Biological Process Level</th>
<th>Gene Count</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Positive regulation of biological process</td>
<td>41</td>
<td>0.000000026</td>
</tr>
<tr>
<td>2) Positive regulation of cellular process</td>
<td>35</td>
<td>0.00000034</td>
</tr>
<tr>
<td>3) Positive regulation of signal transduction</td>
<td>13</td>
<td>0.0000039</td>
</tr>
<tr>
<td>4) Apoptosis</td>
<td>32</td>
<td>0.000016</td>
</tr>
<tr>
<td>5) Programmed cell death</td>
<td>32</td>
<td>0.000018</td>
</tr>
<tr>
<td>6) G-protein coupled receptor signaling pathway</td>
<td>51</td>
<td>0.000048</td>
</tr>
<tr>
<td>7) Cell surface receptor linked signal transduction</td>
<td>67</td>
<td>0.00013</td>
</tr>
<tr>
<td>8) Signal transduction</td>
<td>111</td>
<td>0.00054</td>
</tr>
<tr>
<td>9) Cell communication</td>
<td>116</td>
<td>0.0016</td>
</tr>
<tr>
<td>10) Organismal physiological process</td>
<td>84</td>
<td>0.0052</td>
</tr>
</tbody>
</table>

**Table 2**: A list of statistically-significant GO terms representing the greatest number of affected genes for both the up- and down-regulated lists.

<table>
<thead>
<tr>
<th>GO Term: Biological Process Level</th>
<th>Gene Count</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Regulation of biological process</td>
<td>117</td>
<td>0.0018</td>
</tr>
<tr>
<td>2) Regulation of cellular process</td>
<td>112</td>
<td>0.0011</td>
</tr>
<tr>
<td>3) Regulation of physiological process</td>
<td>104</td>
<td>0.011</td>
</tr>
<tr>
<td>4) Cell communication</td>
<td>104</td>
<td>0.014</td>
</tr>
<tr>
<td>5) Cell communication</td>
<td>116</td>
<td>0.0016</td>
</tr>
<tr>
<td>6) Signal transduction</td>
<td>111</td>
<td>0.00054</td>
</tr>
<tr>
<td>7) Response to stimulus</td>
<td>87</td>
<td>0.015</td>
</tr>
<tr>
<td>8) Organismal physiological process</td>
<td>84</td>
<td>0.0052</td>
</tr>
</tbody>
</table>

Tables one and two provide a more global view of the engineered tissue’s genetic response: Statistically, the bio-chelated mineral solution stimulates biological and cellular processes, while shifting communication patterns away from cell surface receptors and stimulus responses.
Finally, remembering that the project concerns a topically-applied product, the data can be grouped into functional categories supporting familiar skin care claims. For instance, the three KEGG pathways identified from the up-regulated list can be combined with a few miscellaneous genes under the heading "Barrier Function". It is these categories, supported by a number of genes similar to those delineating KEGG pathways, which provide a starting place for subsequent investigations. This data suggests that the blend of five bio-chelated minerals positively impacts barrier function, functions as an anti-inflammatory, protects against apoptosis, and soothes the skin. Appendix 1 contains a list of the gene symbols, their names, the ratios of treated to untreated expression, and brief descriptions for the four functional groupings. These four categories have the greatest support in terms of the number of related genes and are thus the most likely to manifest in further in-vitro or in-vivo studies.

Discussion

Data from DNA microarrays is notoriously difficult to interpret. The volume of information generated is daunting. There is little known about many of the genes included and much less about their role in skin biology. Further, such data is only a starting point to illuminate opportunities for follow-up studies and validation.

Fortunately, the software available to assist in the analysis provides a good start. For example, the genes found to be up-regulated in the KEGG pathways indicate a positive impact on skin’s protective properties. In contrast, UVB exposure represses genes involved with cell adhesion, and an acetone treatment down-regulates genes tied to both cell adhesion and tight junctions [7, 8]. Where acetone disrupts barrier function, this mineral blend should enhance it.

The down-regulation of so many neuroactive receptors and the MAPK pathway is a strong indication of anti-inflammatory properties. The G-protein coupled receptors trigger secondary messenger systems though cAMP or MAPK pathways, and inflammation is often the result [9, 10, and 11]. Many also have an effect on barrier homeostasis through modulation of intercellular calcium levels [12, 13]. By down-regulating these receptors, the bio-chelated minerals should moderate skin’s response to stress and reduce inflammation. For example, green tea’s soothing and reparative benefits are due, in part, to the inhibition of MAPK phosphorylation [14].

The results pointing to a measurable affect on apoptosis are more difficult to interpret. It appears that this mineral blend reduces apoptosis. This may be due to the muting of stress responses through the receptor signaling and MAPK pathways [15, 16]. The bio-chelated mineral’s effect on cell proliferation and differentiation is less clear. Growth factors EREG, (Epiregulin), IGF1 (Insulin-like growth factor), IRS1 (Insulin receptor substrate 1) and NUDT6 (Nudix-type motif 6) are all up-regulated. It does appear that cell proliferation is up-regulated, but more data is required to verify this and any potential changes in cell differentiation. Given the number of affected genes related to enhanced barrier function and stress responses, perhaps the need for apoptosis is being reduced through reduced cellular stress and damage [17]. Skin disorders marked by inflammation and increased apoptosis might provide an interesting area for further study [18].
Finally, a noteworthy subset of regulated genes can be grouped under the heading of generalized stress response. Combined with the neuroactive receptor and MAPK categories, skin's response to stress is surely influenced by the topically applied mineral blend. It would be interesting to see how treated tissue models respond to various stresses in future studies.

It is clear that bio-available minerals have an impact when applied topically. The cosmetic industry's claims that minerals can relax the skin and have anti-inflammatory properties are supported by this data.

References

References

Appendix 1: Sampling of the genes modified by a blend of five bio-chelated minerals. The ratio of the fluorescent intensity of the treated versus untreated sample is given along with a brief description of its known function. Ratios greater than one indicate the gene was up-regulated and those less than one denote a down-regulation. Categories were chosen based on the numbers of genes thought to influence cosmetically-relevant functions.

**Barrier Function**

1) PPP2R1A; Protein Phosphatase 2 (1.50). Functions as a negative control for cell growth and division and is involved in regulating tight junctions.
2) CLDN5; Claudin 5 (1.46). Encodes for claudin proteins, components of tight junction strands, which provide a barrier to prevent water and solutes from freely passing between epithelial sheets.
3) CLDN7; Claudin 7 (1.37). Up-regulates claudin trans-membrane proteins to seal tight junctions and maintain cell-to-cell adhesion.
4) PARD3; Par-3 Partitioning Defective 3 Homolog (C. Elegans) (1.43). Par-3 is involved in tight junction assembly and cell polarization.
5) EPB41L2; Erythrocyte Membrane Protein Band 4.1-like 2 (1.60). Part of the family of tight junction-associated proteins that cross-link and anchor tight junction strand proteins to the cytoskeleton.
6) SPK; Symplekin (1.43). A protein found in cytoplasmic plaques of tight junctions.
7) CMG2; Capillary Morphogenesis Protein-2 (1.35). A collagen type IV and laminin binding protein that plays a role in basement membrane assembly and synthesis.
8) CNTNAP2; Contactin Associated Protein-like 2 (1.44). Mediates the adhesion of cells to other cells or to the extracellular matrix.
9) ITGAL; Integrin alpha L subunit (1.41). Interacts with intercellular adhesion molecule 1 (ICAM1) to increase cell-to-cell adhesion.
10) ECM2; Extracellular Matrix Protein 2 (1.35). Thought to play a role in cell-matrix adhesion.
11) BGN; Biglycan (1.49). A proteoglycan found in the extracellular matrix that interacts with collagen.
12) PPL; Periplakin (1.49). A protein that forms part of the scaffold onto which the cornified envelope is assembled.
13) FABP5; Epidermal Fatty Acid Binding Protein (1.55). Associated with the restoration of barrier function and keratinocyte differentiation.
14) ACHE; Acetyl cholinesterase (YT Blood Group) (1.7). Mediates cell-to-cell adhesion and interactions and intracellular signaling.
15) AGPAT1; 1-Acylglycerol-3-Phosphate O-Acyltransferase 1 (Lysophosphatidic Acid Acyltransferase, alpha) (1.44). Involved in the synthesis of glycerophospholipids and triglycerides to maintain cellular integrity and provide energy for cellular functions.
**Anti-Inflammation**

1) **ALOX5AP;** Arachidonate 5-Lipoxygenase-activating Protein (0.60). Binds to arachidonic acid and may activate 5-lipoxygenase. Has a role in the inflammation response.

2) **C4B;** Complement Component 4B (0.58). Participates in inflammatory and immune responses.

3) **CTSE;** Cathepsin E (1.50). This proteinase degrades the pro-inflammatory IL-18 and IL-1 beta. A deficiency is thought to trigger atopic dermatitis.

4) **EREG;** Epiregulin (1.39). An epidermal growth factor produced by keratinocytes that down-regulates IL-18 to reduce inflammation. A deficiency is tied to chronic dermatitis.

5) **LTC4S;** Leukotriene C4 Synthase (0.68). A synthase that forms a pro-inflammatory mediator.

6) **MAPK11;** Mitogen-activated Protein Kinase 11 (0.62). Activates downstream targets in response to inflammation and stress.

7) **MEFV;** Pyrin/Marenostrin (0.58). Involved in the inflammatory response and may play a role in apoptosis.

8) **PLA2G2A;** Group IIA Phospholipase A2 (0.56). Secreted member of the phospholipase family that plays a role in inflammation, host defense, and phospholipid metabolism.

9) **PLA2G6;** Phospholipase A2 group VI Cytosolic Calcium-independent (0.65). Stimulates the pro-inflammatory precursor arachidonate and is involved in cell cycle control, cell proliferation, and apoptosis. Tied to the MAPK pathway.

10) **DRD4;** Dopamine D4 Receptor (0.61). Found to cause erythema, swelling and a decrease in keratinocyte mitosis. Said to inhibit adenylyl cyclase and thus the signal transducer cAMP. Also involved in the activation of the MAPK pathway.

11) **A 23 P412990/was HRH3;** was Histamine H3 Receptor (0.57). Causes inflammation and works via the PKC and MAPK pathways. Stimulates production of basic fibroblast and nerve growth factors. Inflammation disorders sensitive to stress strongly express histamine receptors.

12) **HTR1B;** 5-hydroxytryptamine (serotonin) 1B Receptor (0.61). Inhibits adenylyl cyclase and thus cAMP levels. It is tied to inflammation, edema and itching. It stimulates fibroblast cell proliferation.

13) **OPRL1;** Opiate Receptor-like 1 (0.54). Inhibits adenylyl cyclase and thus cAMP. The agonist is elevated in dermatitis and psoriasis and is associated with itching. UVR, IL-1 alpha, and DAG stimulate keratinocytes to produce the agonist beta-endorphin.

14) **CRHR2;** Corticotrophin-Releasing Hormone Receptor 2 (0.51). Activates adenylyl cyclase and increases level of intracellular calcium ions. Tied to inflammation.

15) **UCN/SPC;** Urocortin 3/Stresscopin (0.69). Ligand for CRH receptor 2. Activates the MAPK pathway and inhibits keratinocyte proliferation.
16) GPR7; G Protein-coupled Receptor 7/Neuropeptide b/w (0.54). Inhibits adenyl cyclase and stimulates intercellular calcium. Tied to inflammatory and immune responses and can interact with opioid ligands.

17) LTB4R2; Leukotriene b4 (0.67). Inhibits adenyl cyclase and stimulates calcium mobilization. The ligand is derived from arachidonic acid and is a potent mediator of inflammation. It is a chemoattractant and induces chemotaxis. The receptor also activates the phosphatidylinositol-calcium second messenger system.

18) TBXA2R; Thromboxane 2A Receptor (0.66). Activates the phosphatidylinositol-calcium second messenger system and increases intracellular calcium levels. The ligand is derived from arachidonic acid. It is tied to itching and oxidative stress.

19) SSTR3; Somatostatin Receptor 3 (0.54). Inhibits adenyl cyclase and thus cAMP. Known to affect the basal secretion of histamine and immune reactions. Modulates cell growth and cell-matrix adhesion.

**Apoptosis**

1) BCAP31; B-cell Receptor-associated Protein 31 (0.63). Apoptosis-inducing endoplasmic reticulum protein.
2) CIDEB; Cell Death-inducing DFFA-like Effector b (0.43). Induces apoptosis and regulates the nuclease activity of DNA fragmentation factors DFFA and DFFB.
3) DEDD2; Death Effector Domain-containing DNA Binding Protein 2 (0.56). Induces apoptosis.
4) DEFCAP; Death Effector Filament-forming ced-4-like Apoptosis Protein (0.68). Induces apoptosis.
5) DFFA; DNA Fragmentation Factor (0.69). Degrades DNA during apoptosis.
6) GADD45B; Growth Arrest & DNA Damage-inducible 45 beta (0.67). Participates in TGF-beta-induced apoptosis by acting upstream of p38 activation and inhibits cell growth. Up-regulated by UV radiation.
7) GALR3; Galanin Receptor 3 (0.42). Inhibits adenyl cyclase and induces apoptosis.
8) TNFRSF5; Tumor Necrosis Factor Receptor Superfamily member 5 (1.62). Inhibits apoptosis and stimulates B-cell proliferation.
9) TNFRSF9; Tumor Necrosis Factor Receptor Superfamily member 9 (0.41). Induces apoptosis, controls cell proliferation, and regulates immune responses.
10) YARS; Tyrosyl-tRNA Synthetase (0.68). Secreted during apoptosis and then is cleaved to form IL-8 and a chemotactic factor.
11) CD19; CD19 Antigen (1.72). Member of the immunoglobulin superfamily that inhibits apoptosis and enhances B-cell receptor responses.
12) FLJ23467; Protein similar to human GG2-1 (1.47). TNF-induced protein that inhibits apoptosis and may play a role in inflammation.
13) IGF1; Insulin-like Growth Factor 1 (1.56). Stimulates cell proliferation and inhibits apoptosis.
14) IRS1; Insulin Receptor Substrate 1 (1.51). This docking protein mediates IGF1 signaling and inhibits apoptosis.
15) PSMB9; Proteasome subunit beta type 9 (1.31). Cytokine-induced and acts in antigen processing and apoptosis inhibition.
General Stress Response

1) ATM; Ataxia Telangietasia Mutated (1.46). A protein kinase that activates key proteins in the DNA damage checkpoint. It is involved with DNA stability and apoptosis.
2) CSNK1E; Casein Kinase 1 epsilon (1.53). May have a function in DNA repair and the circadian rhythm.
3) FKBPL; FK506-binding Protein-like (1.38). Modulates epidermal growth factor receptor function and is thought to be a heat shock protein involved in cell cycle control and radiation response.
4) HSF1; Heat Shock Transcription Factor 1 (1.49). Activates heat shock proteins, triggers apoptosis, and reduces oxidative stress.
5) HSPH1; Heat Shock Protein (105kDa) (1.76). Provides heat resistance and maintains and repairs protein folds.
6) LOC20447; (1.31). A protein anti-oxidant member of the thioredoxin family.
7) MT1A; Metallothionein 1A (1.46). Heavy metal binding protein that protects against heavy metal toxicity and is thought to have anti-oxidant properties.
8) RPS6KA1; Ribosomal Protein S6 Kinase 1 (1.59). Responses to various stress signals and is thought to inhibit apoptosis.
9) SERPINA6; Serine Proteinase Inhibitor clade A member 6 (0.57). A plasma steroid binding glycoprotein that responses to stress.
10) MADD; MAPK Activating Death Domain (0.69). Stimulates MAPK signaling pathways and blocks apoptosis in neuronal cells undergoing cytotoxic stress. Also has an essential role in calcium-dependent neurotransmitter release.
11) MTA2; Metallothionein 2A (0.69). Heavy metal binding protein that functions in metal homeostasis, stress response, and cell proliferation. May play a role in apoptosis.
12) MTF1; Metal Response Element Binding Transcription Factor 1 (0.58). A transcriptional activator that may play a role in stress and heavy metal responses.
13) DEFB119; Homo Sapiens Defensin beta 119 (1.52). An anti-microbial protein.
**Tradename:** ACB Bio-Chelate 5 PF

**Code:** 20339PF


**Test Request Form #:** 625

**Lot #:** 28813

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

**Study Director:** Erica Segura

**Principle Investigator:** Meghan Darley

**Test Performed:** Cellular Viability Assay

**Introduction**

The cellular viability assay is useful for quantitatively measuring cell-mediated cytotoxicity, cell proliferation and mitochondrial metabolic activity. Increased metabolism in a cell indicates ample cellular respiration and adenosine triphosphate (ATP) production. ATP is the molecular energy of cells and is required in basic cell function and signal transduction. A decrease in ATP levels indicates cytotoxicity and decreased cell function while an increase in ATP levels indicates healthy cells.

The cellular viability assay was conducted to assess the ability of **ACB Bio-Chelate 5 PF** to increase cellular metabolic activity in cultured dermal fibroblasts.

**Assay Principle**

The assay utilizes a nonfluorescent dye, resazurin, which is converted to a fluorescent dye, resorufin, in response to chemical reduction of growth medium from cell growth and by respiring mitochondria. Healthy cells that are in a proliferative state will be able to easily convert resazurin into resorufin without harming the cells. This method is a more sensitive assay than other commonly used mitochondrial reductase dyes such as MTT. An increase in the signal generated by resazurin-conversion is indicative of a proliferative cellular state.
Materials

A. Kit: PrestoBlue™ Cell Viability Reagent (Invitrogen, A13261)
B. Incubation Conditions: 37°C at 5% CO₂ and 95% relative humidity (RH)
C. Equipment: Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Pipettes
D. Cell Line: Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers: Dulbecco’s Modified Eagle Medium (DMEM); Penicillin-Streptomycin (50U-50mg/mL); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS)
F. Culture Plate: Falcon flat bottom 96-well tissue culture treated plates
G. Reagents: PrestoBlue™ reagent (10X)
H. Other: Sterile disposable pipette tips

Methods

Human dermal fibroblasts were seeded into 96-well tissue culture plates and allowed to grow to confluency in complete DMEM. A 10-fold serial dilution was performed resulting in ACB Bio-Chelate 5 PF concentrations of 0.1% and 0.01% in complete DMEM and incubated with fibroblasts for 24 hours.

Ten microliters of viability reagent was added to 90µL of cell culture media in culture wells and a fluorometric measurement was taken at 560nm for excitation and 590nm for emission.
Results

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

**ACB Bio-Chelate 5 PF** did not exhibit significant effects on the cellular metabolism.

Cellular metabolism results are shown as mean fluorescence units (MFU) and expressed as percentage change, calculated by the below equation:

\[
\text{Percent (\%) Change} = \frac{\text{MFU}_{\text{Control}} - \text{MFU}_{\text{Sample}}}{\text{MFU}_{\text{Control}}} \times 100
\]

![Viability Assay Graph](image)

**Figure 1:** Cellular Metabolism of **ACB Bio-Chelate 5 PF**-treated fibroblasts expressed in terms of percent of control.

Discussion

In this study, **ACB Bio-Chelate 5 PF** (code 20339PF) was tested to evaluate its effects on the viability of normal human dermal fibroblasts (NDHF). At concentrations of both 0.1% and 0.01% **ACB Bio-Chelate 5 PF** (code 20339PF), nor the preservatives contained therein exhibited any inhibition of cell viability. It can therefore be concluded that at normal use concentrations **ACB Bio-Chelate 5 PF** (code 20339PF) is not cytotoxic.