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Office de la Propriété Intellectuelle du Canada

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Canadian Intellectual Property Office

CA 3096081 A1 2019/10/10

(21) 3 096 081

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/04/05
(87) Date publication PCT/PCT Publication Date: 2019/10/10
(85) Entrée phase nationale/National Entry: 2020/10/02
(86) N° demande PCT/PCT Application No.: US 2019/026045
(87) N° publication PCT/PCT Publication No.: 2019/195714
(30) Priorité/Priority: 2018/04/05 (US62/653,021)

(51) Cl.Int./Int.Cl. *A61K 9/00* (2006.01), *A61K 35/74* (2015.01), *A61K 38/17* (2006.01)

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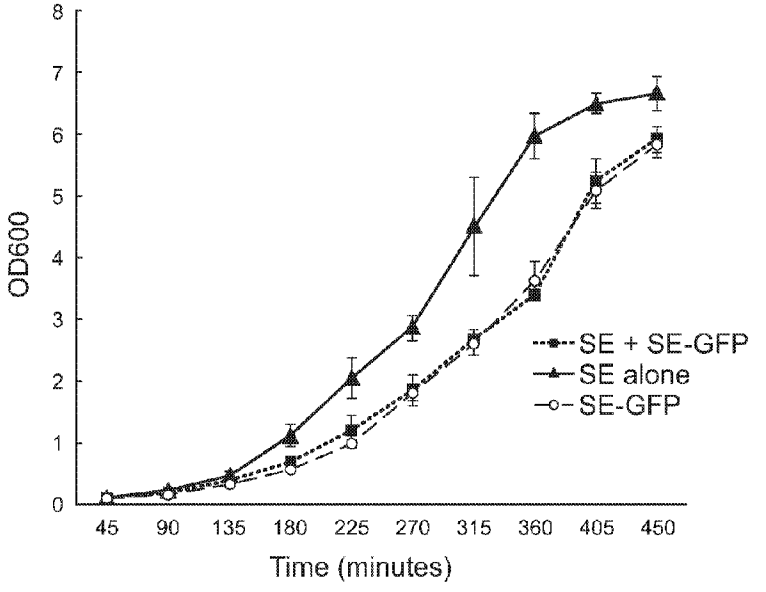
(54) Titre : METHODES ET COMPOSITIONS DE TRAITEMENT DE MALADIES DE LA PEAU A L'AIDE DE MICRO-ORGANISMES RECOMBINANTS

(54) Title: METHODS AND COMPOSITIONS FOR TREATING SKIN DISEASE WITH RECOMBINANT MICROORGANISMS

FIG. 1



(57) Abrégé/Abstract:
The present disclosure provides methods and compositions for treating skin diseases, comprising an engineered microorganism expressing therapeutically relevant recombinant fusion polypeptides. In one embodiment, the disclosure provides a method for treating skin disease, such as atopic dermatitis, comprising administering a to a subject in need thereof a composition comprising live biotherapeutic product (LBP) comprising human filaggrin-secreting Staphylococcus epidermidis. In a further aspect, the present invention provides a pharmaceutical composition comprising a filaggrin polypeptide described herein. Further still, the present invention provides a kit comprising a composition disclosed herein and instructions for use.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 October 2019 (10.10.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/195714 A8

(51) International Patent Classification:
A61K 9/00 (2006.01) *A61K 38/17* (2006.01)
A61K 35/74 (2015.01)

(21) International Application Number:
PCT/US2019/026045

(22) International Filing Date:
05 April 2019 (05.04.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/653,021 05 April 2018 (05.04.2018) US

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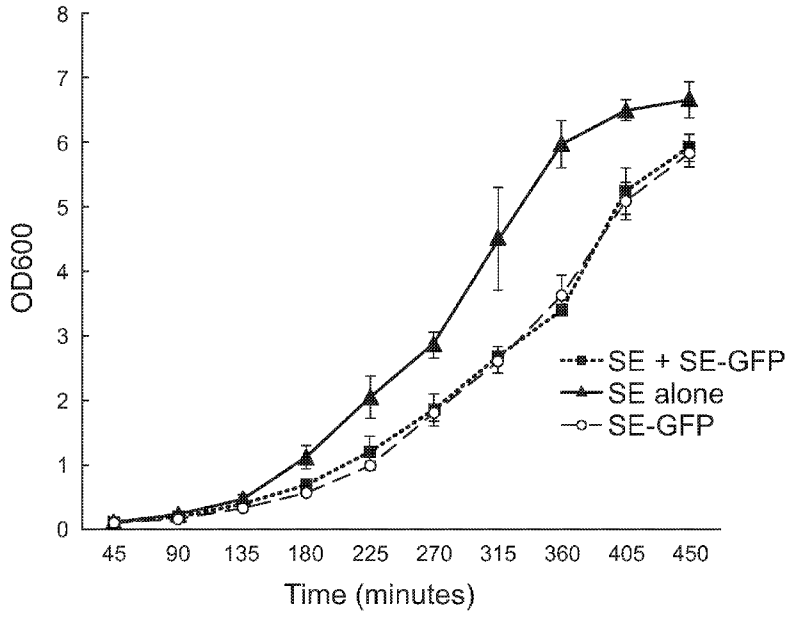
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

WO 2019/195714 A8

(54) Title: METHODS AND COMPOSITIONS FOR TREATING SKIN DISEASE WITH RECOMBINANT MICROORGANISMS

FIG. 1



(57) Abstract: The present disclosure provides methods and compositions for treating skin diseases, comprising an engineered microorganism expressing therapeutically relevant recombinant fusion polypeptides. In one embodiment, the disclosure provides a method for treating skin disease, such as atopic dermatitis, comprising administering a to a subject in need thereof a composition comprising live biotherapeutic product (LBP) comprising human filaggrin-secreting Staphylococcus epidermidis. In a further aspect, the present invention provides a pharmaceutical composition comprising a filaggrin polypeptide described herein. Further still, the present invention provides a kit comprising a composition disclosed herein and instructions for use.

[Continued on next page]

WO 2019/195714 A8

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

(48) Date of publication of this corrected version:
23 July 2020 (23.07.2020)

(15) Information about Correction:
see Notice of 23 July 2020 (23.07.2020)

METHODS AND COMPOSITIONS FOR TREATING SKIN DISEASE WITH RECOMBINANT MICROORGANISMS

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 62/653,021, filed on April, 5, 2018, the entire contents of which are incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

Ichthyosis vulgaris (IV) is a chronic, xerotic, sclay skin disease with an estimated incidence and prevalence of 1 in 250,[1,2] which gives a total patient population of 1.3 million in the United States. Clinical features of IV usually appear at around 2 months of age and include generalized xerosis and fine, white to gray scales that are prominent on the abdomen, chest, and extensor surfaces of the extremities.[3] Some IV patients also experience hypohidrosis and heat intolerance.[4] The pathogenesis of IV has long been identified as a decrease in the size or number, or even a complete absence of, epidermal keratohyaline granules.[5-7] In addition, due to genetic factors, patients with IV are at increased risk for atopic dermatitis (AD), asthma, and allergies.[8]

Icthyosis vulgaris is an autosomal semidominant disease caused by loss-of-function mutations in the gene encoding filaggrin.[9] Filaggrin is an essential structural protein that is derived from profilaggrin, which breaks down into monomeric filaggrin in the stratum corneum and reinforces the skin barrier by binding to keratins and other intermediate filament proteins in the keratinocyte cytoskeleton.[10]

Many studies have identified loss-of-function mutations in *FLG* in IV and atopic dermatitis patients,[11-14] and these mutations are associated with disorganized keratin filaments, skin barrier defects[15] and microfractures in the stratum corneum leading to enhanced percutaneous allergen sensitization.[16-19] Moreover, filaggrin and its breakdown products have significant additional functions in the skin including moisturizing the skin (via hygroscopic amino acids or "natural moisturizing factors"),[20,21] effecting production of antimicrobial molecules (particularly against *S. aureus*),[22] and maintaining both a beneficial lipid profile[23,24] and pH[24-26] in the skin.

Current treatment options for IV include primarily topical water evaporation suppressants (e.g., sodium chloride, urea, lactic acid, salicylic acid), and moisturizers

(e.g., glycerol, propylene glycol, dexpanthenol).[4] Topical retinoids may also be prescribed in an effort to slow the body's production of skin cells; however, as Vitamin A derivatives, long-term use is not ideal. Notably, many patients with IV experience a significantly reduced quality of life,[27-29] due to self-consciousness and social embarrassment, and see a negative impact on domestic life, educational/professional lives, and even leisure/sports activities.[28,29] It is clear that IV is a large, unmet need.

Diverse communities of microorganisms populate the skin, and a square centimeter can contain up to a billion microorganisms.[39] These diverse communities of bacteria, fungi, mites and viruses can provide protection against disease and form dynamic, yet distinct niches on the skin.[40] Increasing evidence has associated altered microbial communities or dysbiosis in the skin with cutaneous diseases,[39,41] especially AD.[42,43] Engineered probiotics are a novel approach based on leveraging the skin microbiome for therapeutic purposes. Notably, an engineered probiotic has important advantages over other methods of drug delivery, as it will establish residence on the patient's skin and continuously and stably deliver therapeutic proteins *in situ*. Furthermore, certain strains of *Staphylococcus epidermidis* (SE) have demonstrated important beneficial immuno-modulatory and anti-pathogen effects in the skin, which are relevant to atopic dermatitis disease phenotype and severity. Moreover, the delivery of filaggrin, which is a structural protein derived from profilaggrin, further enhances the therapeutic approach due to filaggrin's role in the skin barrier and ability to reduce transepidermal water loss and improve skin hydration. The present invention has the surprising advantage of providing methods and compositions for treating skin diseases, *e.g.*, atopic dermatitis, using a genetically engineered, recombinant strain of *Staphylococcus epidermidis* as a skin drug delivery system that secretes human filaggrin to address the pathophysiology of atopic dermatitis (*e.g.*, AZT-01). Once applied to the skin, stable colonization of the skin and the subsequent secretion of filaggrin *in situ* can resolve the disease. The benefits of this invention include its safety as a non-steroidal treatment option, its efficacy due to the invention's combination of benefits from the secretion of filaggrin along with the benefits of the topical application of *Staphylococcus epidermidis*, and its ability to be therapeutically effective at even a low frequency of application (no more than once a day).

The present invention therefore addresses the long-felt need for an effective treatment for inflammatory skin diseases, and in particular, for IV. The present invention

is also one of the first reported demonstrations of commensal skin bacteria that can secrete therapeutic proteins to treat skin disease.

SUMMARY OF THE INVENTION

The present disclosure features a novel treatment modality for skin disease, for example ichthyosis vulgaris (IV), that directly addresses the pathophysiology of ichthyosis vulgaris and consists of a live biotherapeutic product (LBP) comprised of human filaggrin-secreting Staphylococcus epidermidis. A goal of the present disclosure is to supplement the skin with long-term, stable delivery of filaggrin via a beneficial microbe-based chassis system. While the bacteria's potential probiotic properties are leveraged and optimized by the present invention, disease remediation is based on rational targeted expression of relevant proteins that directly address underlying pathophysiology. Additionally, because response to different microbial species and strains will differ between individuals, a modular design to control therapeutic delivery will likely provide significantly improved pharmacokinetics and disease resolution over current approaches that leverage naturally occurring strains. Importantly, the introduction of *S. epidermidis* could aid in re-establishing skin homeostasis by targeting pathogen-driven dysbiosis, as certain strains can secrete antimicrobial peptides.

Thus, the present invention relates, *inter alia*, to methods and compositions for treating skin diseases, for example IV, comprising an engineered microorganism capable of expressing therapeutically relevant recombinant fusion polypeptides (*i.e.* proteins, peptides, or amino acids).

The present invention features, in a first aspect, a recombinant microorganism capable of secreting a polypeptide, wherein the recombinant microorganism comprises an expression vector comprising a first coding sequence comprising a gene capable of expressing the polypeptide and a second coding sequence comprising a gene capable of expressing a cell penetrating peptide. In a related embodiment, the recombinant microorganism further comprising a third coding sequence comprising a gene capable of expressing an export signal. In yet another embodiment, the expression of the first coding sequence, second coding sequence and third coding sequence is under the control of a promoter. In other embodiments, the arrangement of the first coding sequence, second coding sequence and third coding sequences are in-frame. In yet another related embodiment, the first coding sequence, second coding sequence and third coding

sequence are operably linked to a promoter. In one embodiment, the recombinant microorganism is bacteria, or a combination of bacteria. In another embodiment, the polypeptide is filaggrin, or a variant thereof. In other embodiments, the microorganism is selected from the group consisting of *Bifidobacterium*, *Brevibacterium*, *Propionibacterium*, *Lactococcus*, *Streptococcus, Staphylococcus, Lactobacillus, Enterococcus, Pediococcus, Leuconostoc, or Oenococcus*, or combinations thereof. In other embodiments, the recombinant microorganism is *Staphylococcus epidermidis*. In some embodiments, the microorganism secretes a filaggrin fusion protein. In a further embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In one embodiment, the filaggrin fusion protein consists of SEQ ID NO: 1.

The present invention features, in a further aspect, a method for producing a live biotherapeutic composition, the method comprising (a) transfecting a cell with (i) a first coding sequence comprising a nucleic acid sequence capable of expressing a therapeutic polypeptide, and (ii) a second coding sequence comprising a nucleic acid sequence capable of expressing a cell penetrating peptide; and (b) allowing the transfected cell to produce a therapeutic polypeptide fusion protein; and (c) obtaining the live biotherapeutic composition. In a related embodiment, the method further comprises (iii) transfecting the cell with a third coding sequence comprising a nucleic acid sequence capable of expressing an export signal. In another embodiment, the first coding sequence, second coding sequence and third coding sequences are arranged in a single plasmid. In yet another embodiment, the arrangement of the first coding sequence, second coding sequence and third coding sequences are operably linked to a promoter. In other embodiments, the cell is selected from the group consisting of wherein the microorganism is selected from the group consisting of *Bifidobacterium, Brevibacterium, Propionibacterium, Lactococcus, Streptococcus, Staphylococcus, Lactobacillus, Enterococcus, Pediococcus, Leuconostoc, or Oenococcus*, or combinations thereof. In yet another embodiment, the cell is *Staphylococcus epidermidis*. In other embodiments, the therapeutic polypeptide fusion protein is a filaggrin fusion protein, or a variant thereof. In a further embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In one embodiment, the filaggrin fusion protein consists of SEQ ID NO: 1.

In another aspect, the disclosure features a composition comprising a filaggrin polypeptide, or a variant thereof. In one embodiment, the filaggrin polypeptide is a fusion protein. In a further embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In one embodiment, the filaggrin fusion protein consists of SEQ ID NO: 1.

The present invention features, in a further aspect, a composition obtained by any one of the method disclosed or described herein. In a related embodiment, the composition comprises a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous solution, an emulsion, a cream, a lotion, a gel, or an ointment.

The present invention features, in a further aspect, a live biotherapeutic composition comprising a recombinant microorganism wherein the recombinant microorganism comprises (i) a first coding sequence comprising a nucleic acid sequence capable of expressing a therapeutic polypeptide; (ii) a second coding sequence comprising a nucleic acid sequence capable of expressing a cell penetrating peptide; (iii) a third coding sequence comprising a nucleic acid sequence capable of expressing an export signal; and (iiv) a promoter operably linked to the first coding sequence, the second coding sequence and the third coding sequence; wherein the first coding sequence, second coding sequence and first coding sequence is capable of expressing a filaggrin fusion protein, or variant thereof. In a further embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In a related embodiment, the recombinant microorganism is *Staphylococcus epidermidis*. In a further embodiment, the export signal exports the filaggrin fusion product, or variant thereof, out of the recombinant microorganism. In yet another embodiment, the cell penetrating peptide facilitates the entry of the filaggrin fusion product, or variant thereof, into a human keratinocyte. In another embodiment, the composition comprises a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous solution, an emulsion, a cream, a lotion, a gel, or an ointment.

The present invention features, in a further aspect, a kit comprising any one of the compositions disclosed or described herein and instructions for use.

The present invention features, in a further aspect, a method of treating a skin disease comprising administering to a subject in need thereof the composition of any one of the compositions disclosed or described herein. In one embodiment, the skin disease is IV. In another embodiment, the skin disease is atopic dermatitis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph that shows characterization of growth strains in liquid culture. SE alone (solid black line, triangle markers) grew more quickly than SE-GFP alone or SE and SE-GFP together.

FIG. 2A-C shows characterization of growth of strains in RHE. (A) SE with empty vector (i.e. no protein production). (B) SE-GFP alone on RHE shows slower growth at 24 hours. (C) when mixed together 50:50 on RHE, SE empty vector outcompetes SE-GFP.

FIG. 3A-C shows fluorescence and reflective overlays. (A) Fluorescent SE on the surface and outlining individual cells in the stratum corneum (dotted line shows outlines of corneocytes as an example). (B-C) Fluorescence and light wavelength overlays of RHE 3.5 hours at 37°C after Dermaroller application followed by topical GFP application, showing the bacteria have localized into the breach of a damaged stratum corneum with no evidence of bacteria in epidermis at that level. Depths taken at 50 μm (B) and 70 μm (C).

FIG. 4A-C shows the results of purifying GFP. Protein was purified from 225 mL culture of SE (2.7-2.9 OD/mL) and lysed with CellB-Lyse (Sigma). 4mL eluate was produced and ran on a Ni-NTA column in a buffer of PBS pH 7.4 and yielded 0.4 mg. (A) InVision His-tag stain on an SDS-PAGE gel with purified protein from SE. Lanes (1) lysate pellet; (2) supernatant, clarified; (3) pass-through; (4) eluate; (5) concentrate; (6) marker; (7) supernatant, clarified; (8) pass-through; (9) eluate; and (10) concentrate. (C) Purified GFP from SE.

FIG. 5A-O shows characterization of the protein with and without the RMR signal using 5 μg GFP as a reporter in RHE. (A-D) Two-photon images of topically applied GFP with (C,D) or without (A,B) the RMR signal at 30 minutes (A,C, E, G) or 60 minutes (B,D,F,H). Images are compiled Z-stacks projected onto a 2D plane. (E-H)

3D surface analysis to examine depths of protein penetration into RHE (I-N) Confocal images of GFP (K, N) GFP + RMR (J,M) or vehicle (I,L) using light (L-N) or fluorescent (I-K) wavelengths. (O) RHE punctured with Dermaroller microneedle with 50 ug GFP-RMR. Images taken 30 minutes after application.

FIG. 6A-E shows preliminary bioinformatics analyses of human filaggrin. (A) homology of human filaggrin domains. (B) sequence alignment of human filaggrin domains. (C) hydrophobicity plot of the human filaggrin domains with a corresponding diagram of the filaggrin sequence. (D) Example SAR exercise for human filaggrin simians 9-10 using a hydrophobicity plot to remove QSGEnSGRnSFLYQVSnHEQSES repeats at the N-terminus of hFLG 9-10 and € resulting sequence of this protein with RMR cell penetrating peptide added. (F) Overview of filaggrin structure. (Top) Profilaggrin and filaggrin gene structure. The majority of filaggrin is encoded by exon 3. (Bottom) Profilaggrin protein structure.

FIG. 7A-E shows assay development. (A-C) Western blots of hFLG in the media isolate from filaggrin-producing SE culture with various antibodies. (A) Sigma polyclonal anti-hFLG antibody. (B) SantaCruz polyclonal anti-hFLG antibody. (C) polyclonal anti-hFLG antibody. (D) Proposed workflow for method development of MS-based filaggrin detection. (E) Identity of expressed human filaggrin from mass spectrometry for hFLG9-10. Colors indicate sequence confidence: dark blue (very high confidence), light blue (observed) and red (not observed).

FIG. 8 shows overview of Biacore surface plasmon resonance (SPR) for filaggrin activity.

FIG. 9A-G shows pilot results of creating standard curves for filaggrin breakdown products, or natural moisturizing factors (NMFs). Raman spectroscopy can detect individual NMF components (A-D). Mass spectroscopy (E-G) is also very sensitive with limit of quantitation (LOQ) in the pictogram to nanogram range.

FIG. 10 shows overview of workflow.

FIG. 11 shows experimental outline of 16S sequencing.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many

of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them below, unless specified otherwise.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

The term "such as" is used herein to mean, and is used interchangeably, with the phrase "such as but not limited to".

As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1 %. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1 , 99.2, 99.3, 99.4, etc.).

As used herein, the terms "carriers", "carrier system" or "vehicles" refer to compatible substances that are suitable for delivering, containing, or "carrying" a pharmaceutical active ingredient or other materials for administration in a topically applied composition to a patient or subject. Carriers useful herein should be pharmaceutically acceptable. Carriers and vehicles useful herein include any such materials known in the art, which are non-toxic and do not interact with other components of the formulation in which it is contained in a deleterious manner. The term "aqueous" refers to a formulation that contains water or that becomes water-containing following application to the skin or mucosal tissue. Further examples of "carriers" include water, lower alcohols, higher alcohols, polyhydric alcohols, monosaccharides, disaccharides, polysaccharides, hydrocarbon oils, fats and oils, waxes, fatty acids, silicone oils, nonionic surfactants, ionic surfactants, silicone surfactants, and water- based mixtures and emulsion-based mixtures of such carriers.

As used herein, the term "engineered bacterial strain," or a “recombinant bacterial strain” refers to a strain of bacteria that has been "genetically modified" or "engineered" by the introduction of DNA prepared outside the organism into the bacterial strain. For example, the introduction of a plasmid containing new genes or other nucleic acid sequence(s) into bacteria will allow the bacteria to express those genes or other nucleic acid sequence(s). Alternatively, the plasmid containing new genes or other nucleic acid sequence(s) can be introduced to the bacteria and then integrated into the bacteria's genome, where the bacteria will express those genes or other nucleic acid sequence(s).

As used herein, the term “host cell” is meant to refer to a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

The term "isolated" for the purposes of the present disclosure designates a biological material (cell, nucleic acid or protein) that has been removed from its original environment (the environment in which it is naturally present). For example, a polynucleotide present in the natural state in a plant or an animal is not isolated, however the same polynucleotide separated from the adjacent nucleic acids in which it is naturally present, is considered "isolated."

An "isolated nucleic acid molecule” (such as, for example, an isolated promoter) is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regard to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid molecule is free of sequences which naturally flank the nucleic acid molecule in the genomic DNA of the organism from which the nucleic acid molecule is derived.

As used here, the term “genetic element” is meant to refer to a polynucleotide comprising a region that encodes a polypeptide or a polynucleotide region that regulates replication, transcription or translation or other processes important to expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression. Genetic elements may be comprised within a vector that replicates as an episomal element; that is, as a molecule physically independent of the host cell genome. They may be comprised within plasmids. Genetic elements also may be comprised within a host cell

genome; not in their natural state but, rather, following manipulation such as isolation, cloning and introduction into a host cell in the form of purified DNA or in a vector, among others.

As used here, the term “live biotherapeutic product” (or LBP) refers to a product candidate(s) containing bacteria, yeast, and/or other microorganisms.

As used herein, the terms "patient" or "subject", refers to a human or animal (in the case of an animal, more typically a mammal such as domesticated mammals, or animals such as poultry animals and fish and other seafood or freshwater food creatures), that would be subjected to the treatments and compositions of the present invention.

As used herein, the expression "a subject in need thereof" means a human or non-human animal that exhibits one or more symptoms or indicia of atopic dermatitis, and/or who has been diagnosed with IV.

As used herein, the phrase "pharmaceutically acceptable" refers to those active compounds, materials, engineered bacterial strain or strains, compositions, carriers, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications, commensurate with a reasonable benefit/risk ratio.

As used herein, the term “polynucleotide(s)” generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded, or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term polynucleotide includes DNAs or RNAs as

described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia. The term polynucleotide also embraces short polynucleotides often referred to as oligonucleotide(s). "Polynucleotide" and "nucleic acid" are often used interchangeably herein.

As used herein, the terms “polypeptide” or “protein” refer to biological molecules, or macromolecules composed of amino-acid residues bonding together in a chain. The definition of polypeptides used herein is intended to encompass proteins (generally higher molecular weight) composed of one or more long chains of amino acid residues and small peptides (generally lower molecular weight) of a few amino acids. In other embodiments, a single amino acid, although not technically a polypeptide, is also considered within the scope of the disclosure.

As used herein, the term “preventing”, refers to completely or almost completely stopping an abnormal skin condition (e.g. IV) from occurring, for example when the patient or subject is predisposed to an abnormal skin condition or at risk of contracting an abnormal skin condition. Preventing can also include inhibiting, i.e. arresting the development, of an abnormal skin condition.

As used herein, a “promoter” is meant to refer to a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5′ region of a gene, proximal to the transcriptional start site of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. For example, a promoter may be regulated in a tissue-specific manner such that it is only active in transcribing the associated coding region in a specific tissue type(s).

As used herein, the term “reducing the risk of”, refers to lowering the likelihood or probability of an abnormal skin condition (e.g. IV) from occurring, for example when the patient or subject is predisposed to an abnormal skin condition or at risk of

contracting an abnormal skin condition.

As used herein, the term "therapeutically effective amount" refers to an amount of a pharmaceutical active compound, or a combination of compounds, or an amount of pharmaceutical active compound delivered by an engineered bacterial strain or strains, for example a skin treatment agent or agents, when administered alone or in combination, to treat, prevent, or reduce the risk of a disease state or condition, for example an abnormal skin condition (e.g. IV). The term also refers to an amount of a pharmaceutical composition containing an active compound or combination of compounds or an engineered bacterial strain or strains that delivers a pharmaceutical active compound. For example, an effective amount refers to an amount of the compound or an amount of the compound delivered by an engineered bacterial strain or strains present in a formulation given to a recipient patient or subject sufficient to elicit biological activity, for example, activity for treating or preventing an abnormal skin condition.

As used herein, the term "treating" refers to providing a therapeutic intervention to cure or ameliorate an abnormal skin condition (e.g. IV

COMPOSITIONS

The present invention provides skin-colonizing microorganisms, *e.g.*, bacteria, that are genetically altered to express recombinant therapeutic polypeptides for the treatment or prevention of skin disease (Figure 2). Using genetically engineered protein-producing microorganisms, *e.g.*, bacteria, has several advantages over the prior art method of treating skin disease. Therapeutic proteins are able to treat the underlying cause of defects leading to the skin condition. Further, microorganisms, *e.g.*, bacteria, are able to self-replicate while retaining the inserted nucleic acid (*e.g.*, a gene) to continuously produce the therapeutic protein.

The present invention provides skin-colonizing microorganisms, *e.g.*, bacteria, such as for example, *Staphylococcus epidermidis*, that are genetically altered to express therapeutic proteins, *e.g.*, human filaggrin. Using genetically engineered filaggrin-producing microorganisms, *e.g.*, bacteria, has several advantages over using filaggrin supplementation. First, microorganisms, *e.g.*, bacteria, are able to self-replicate while retaining the inserted filaggrin nucleic acid sequence (*e.g.*, a gene). Second, *S. epidermidis* is normally present on the skin and has been shown to inhibit growth of

Staphylococcus aureus, a bacterial species of the same genre that dominates the skin flora in atopic dermatitis flares.

Bacterial Strains

The present invention provides genetically altered microorganisms, *e.g.*, bacteria, capable of expressing recombinant therapeutic proteins. A wide range of microorganisms are suitable for use in the present invention. Examples include, but are not limited to, non-pathogenic and commensal bacteria. Bacteria suitable for use in the present invention include, but are not limited to, Bifidobacterium, Brevibacterium, Propionibacterium, Lactococcus, Streptococcus, Staphylococcus (*e.g.*, S. epidermidis), Lactobacillus (*e.g.*, L. acidophilus), Pediococcus, Leuconostoc, or Oenococcus. In certain embodiments of the invention, the bacterium is *Staphylococcus epidermidis*. In preferred embodiments of the invention, the strain of *S. epidermidis* to be used is incapable of producing biofilms. One such example of a strain of *S. epidermidis* incapable of producing biofilms is *S. epidermidis* strain ATCC 12228. However, in yet other embodiments of the invention, other related or similar species found on the skin can be used.

Therapeutic Proteins

The present invention provides genetically altered microorganisms, *e.g.*, bacteria, capable of expressing recombinant therapeutic proteins. In preferred embodiments of the invention, the therapeutic protein comprises human filaggrin. Human filaggrin is expressed by a human gene encoding filaggrin (*FLG*). Filaggrin is a protein produced by differentiating keratinocytes and functions to aggregate keratin filaments into a cytoskeleton, which, in combination with other components, comprises the cornified cell envelope. *FLG* is a large gene located on chromosome lq21 that produces profilaggrin, an insoluble polyprotein that is proteolyzed to release functional filaggrin monomers (Armengot-Carbo et al. 2014). The therapeutic protein (and, *i.e.*, the gene from which the protein is expressed) of the invention may be from any mammal. Non-limiting examples include, but are not limited to, mouse, rat, rabbit, goat, sheep, horse, cow, dog, primate, or human gene sequence. In preferred embodiments of the invention, the therapeutic protein comprises a recombinant fusion protein comprising filaggrin operably linked to a cell penetrating protein (CPP). In one embodiment, the cell

penetrating protein is RMRRMRRMRR. In other embodiments of the invention, the therapeutic protein comprises a recombinant fusion protein comprising filaggrin operably linked to an export or secretion signal, which allows the recombinant filaggrin to be exported out of the microorganism (*e.g.*, bacteria). In another embodiment, the therapeutic protein comprises a recombinant fusion protein comprising filaggrin operably linked to a cell penetrating protein (CPP) and to an export or secretion signal.

In one embodiment, the fusion protein comprising filaggrin operably linked to a cell penetrating protein is shown in SEQ ID NO. 1

SEQ ID NO. 1

```
                                                      1430        1440
                                                       MQ  SGESSGRSRS
      1450        1460        1470        1480        1490        1500
FLYQVSSHEQ  SESTHGQTAP  STGGRQGSRH  EQARNSSRHS  ASQDGQDTIR  GHPGSSRGGR
      1510        1520        1530        1540        1550        1560
QGSYHEQSVD  RSGHSGYHHS  HTTPQGRSDA  SHGQSGPRSA  SRQTRNEEQS  GDGSRHSGSR
      1570        1580        1590        1600        1610        1620
HHEPSTRAGS  SRHSQVGQGE  SAGSKTSRRQ  GSSVSQDRDS  EGHSEDSERR  SESASRNHYG
      1630        1640        1650        1660        1670        1680
SAREQSRHGS  RNPRSHQEDR  ASNGHSAESS  RQSGTRHAET  SSGGQAASSQ  EQARSSPGER
      1690        1700        1710        1720        1730        1740
HGSRHQQSAD  SSTDSGTGRR  QDSSVVGDSG  NRGSSGSQAS  DSEGHSEESD  TQSVSAHGQA
      1750        1760        1770
GPHQQSHQES  TRGQSGERSG  RSGSFLYQVS  THEQSESRMR  RMRRMRR
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In one embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In one embodiment, the filaggrin fusion protein consists of SEQ ID NO: 1.

In some aspects, the therapeutic protein is not expressed by a microorganism (e.g. a bacteria). In some aspects, the therapeutic protein comprises a filaggrin polypeptide, or a variant thereof. In one embodiment, the filaggrin polypeptide is a fusion protein. In a further embodiment, the filaggrin polypeptide comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1. In one embodiment, the filaggrin fusion protein consists of SEQ ID NO: 1.

Secretion signals

Secretion signals or export signals are peptide sequences on a protein that facilitate the export of the protein through the secretory pathway, which ultimately

results in the protein being secreted from the cell. In the present invention, any secretion signal that facilitates the export of a protein, such as a protein comprising filaggrin, out of a microorganism (*e.g.*, a bacterial cell) is contemplated as a secretion signal.

Cell penetrating peptides

A cell penetrating peptide is a peptide sequence that facilitates or mediates the delivery of a biomolecule (*e.g.*, a protein) in vivo without using any receptors and without causing any significant membrane damage. Cell penetrating peptides that facilitate entry into the skin keratinocytes are contemplated as a cell penetrating peptides of the present invention.

In one embodiment, the cell penetrating protein is RMRRMRRMRR (SEQ ID NO. 2).

Genetic Construct

The present invention utilizes standard molecular biology techniques, *e.g.*, those described in (Sambrook et al. 2001). An example of the genetic construct used for this invention is pAZT, which is based on pJB38, an allelic exchange E. coli-staphylococcal shuttle vector, further comprising additional design features on the plasmid to improve functionality (Bose, J.L., *et al. Applied and environmental microbiology.* 2013;79(7):2218-2224). The plasmid is constructed by inserting cDNA of a gene encoding a therapeutic protein into a restriction site , using standard molecular biology techniques (Figure 2). The insert further comprises a coding sequence driven by a promoter. Such a promoter can be either constitutive or inducible. Examples of inducible promoters include those that are activated by chemical compounds such as alcohols, sugars, metals, or tetracycline, or by physical factors such as light or high temperatures.

The mRNA sequence of human FLG has a Genebank accession No. NM_002016. A plasmid pAZT was constructed by inserting part of the *FLG* cDNA into a restriction site of pJB38. The insert contains a nucleic acid coding sequence driven by a promoter. The construct further comprises a nucleic acid sequence encoding a secretion signal and a cell penetrating peptide, thus resulting in a recombinant filaggrin fusion protein.

Uses of recombinant bacterial strain

It will be understood that the skin disease to be treated can be any disease or disorder associated with skin. In one embodiment the disorder is selected from the group consisting of atopic dermatitis, psoriasis, acne, allergic contact dermatitis, epidermolytic hyperkeratosis, seborrheic dermatitis, eczema, dry skin, allergy, rashes, UV-irritated skin, detergent irritated skin (including irritation caused by enzymes and compounds used in washing detergents and sodium lauryl sulfate), thinning skin (e.g. skin from the elderly and children), bullous pemphigoid, pemphigus vulgaris, impetigo, vitiligio, baldness, and hirsutism. In one particular embodiment, the skin disease is Ichthyosis vulgaris (IV).

Examples of proteins that can be administered according to the invention are preferably eukaryotic proteins. These proteins include, but are not limited to, single amino acids, small peptides, and large proteins. More particularly, genes encoding proteins that are useful in the invention as recombinant therapeutic proteins include, but are not limited to, the following: members of the interleukin family of genes, including, but not limited to, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14 and IL-15 and genes encoding receptor antagonists thereof. Genes which encode hematopoietic growth factors, including but not limited to, erythropoietin, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, macrophage colony stimulating factor, stem cell factor, leukemia inhibitory factor and thrombopoietin are also contemplated in the invention. Genes encoding neurotropic factors are also contemplated, including but not limited to, nerve growth factor, brain derived neurotropic factor and ciliary neurotropic factor. In addition, genes which encode interferons, including, but not limited, to IFN-alpha, IFN-beta and IFN-gamma are included. Further contemplated in the present invention are genes encoding chemokines such as the C-C family and the C-X-C family of cytokines, genes encoding hormones, such as proinsulin and growth hormone, and genes encoding thrombolytic enzymes, including tissue plasminogen activator, streptokinase, urokinase or other enzymes such as trypsin inhibitor. The invention further includes genes which encode tissue repair factors, growth and regulatory factors including, but not limited to, oncostatine M, platelet-derived growth factors, fibroblast growth factors, epidermal growth factor, hepatocyte growth factor, bone morphogenic proteins, insulin-like growth factors, calcitonin and transforming growth factor alpha and beta. Further contemplated

genes include genes encoding structural proteins including filaggrin, actin, collagen, fibrillin, elastin, or scleroprotein.

In particular embodiments, the gene is a gene encoding filaggrin.

Antibodies

Also provided in the present disclosure is an antibody against an epitope of hFLG that is common across all pairs of subdomains. In certain embodiments, the antibody is a chicken IgY.

FORMULATIONS

A formulation for use according to the present invention may comprise any pharmaceutically effective amount of a genetically engineered microorganism, *e.g.*, bacteria, to produce a therapeutically effective amount of a desired polypeptide, for example, at least about 0.01%, about 0.05%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1.0%, about. 1.5%, about 2.0%, about 3.0%, about 4.0%, about 5.0%, about 6.0%, about 7.0%, about 8.0%, about 9.0%, about 10.0%, about 11.0%, about 12.0%, about 13.0%, about 14.0%, about 15.0%, about 16.0%, about 17.0%, about 18.0%, about 19.0%, about 20.0%, about 25.0%, about 30.0%, about 35.0%, about 40.0%, about 45.0%, about 50.0% or more by weight of the genetically engineered microorganism, *e.g.*, bacteria,, the upper limit of which is about 90.0% by weight of the genetically engineered microorganism, *e.g.*, bacteria.

In an alternative embodiment, the formulation for use according to the present invention can comprise, for example, at least about 0,01% to about 30%, about 0.01% to about 20%, about 0.01% to about 5%, about 0.1 % to about 30%, about 0.1% to about 20%, about 0.1% to about 15%, about 0.1 % to about 10%, about 0.1% to about 5%, about 0.2% to about 5%, about 0,3% to about 5%, about 0.4% to about 5%, about 0.5% to about 5%, about 1% to about 5%, or more by weight of a genetically engineered microorganism, *e.g.*, bacteria.

In some aspects, a formulation for use according to the present invention may comprise any pharmaceutically effective amount of a filaggrin polypeptide, or a variant thereof. In one embodiment, the filaggrin polypeptide is a fusion protein. In a further embodiment, the filaggrin polypeptide comprises an amino acid sequence that is 85%,

86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1.

In some embodiments, the formulation is a topical formulation. The topical formulation for use in the present invention can be in any form suitable for application to the body surface, such as a cream, lotion, sprays, solution, gel, ointment, paste, plaster, paint, bioadhesive, suspensions, emulsions, or the like, and/or can be prepared so as to contain liposomes, micelles, and/or microspheres. Such a formulation can be used in combination with an occlusive overlayer so that moisture evaporating from the body surface is maintained within the formulation upon application to the body surface and thereafter. The formulation can include a living biotherapeutic composition and can comprise at least one a genetically engineered microorganism, *e.g.*, an engineered bacterial strain, that produces a recombinant polypeptide. This engineered living biotherapeutic composition can deliver the polypeptide directly to the skin for treating or preventing abnormal skin conditions, and/or skin diseases (*e.g.*, inflammatory skin diseases).

Topical formulations include those in which any other active ingredients are dissolved or dispersed in a dermatological vehicle known in the art, *e.g.* aqueous or nonaqueous gels, ointments, water-in-oil or oil-in-water emulsions. Constituents of such vehicles may comprise water, aqueous buffer solutions, non-aqueous solvents (such as ethanol, isopropanol, benzyl alcohol, 2-(2-ethoxyethoxy)ethanol, propylene glycol, propylene glycol monolaurate, glycofurol or glycerol), oils (e.g. a mineral oil such as a liquid paraffin, natural or synthetic triglycerides such as Miglyol™, or silicone oils such as dimethicone). Depending upon the nature of the formulation as well as its intended use and site of application, the dermatological vehicle employed can contain one or more components (*e.g.*, when the formulation is an aqueous gel, components in addition to water) selected from the following: a solubilizing agent or solvent (e.g. a β-cyclodextrin, such as bydroxypropyl β- cyclodextrin, or an alcohol or polyol such as ethanol, propylene glycol or glycerol); a thickening agent (e.g. hydroxyethylceliulose, hydroxypropylcellulose, carboxymethylcellulose or carbomer); a gelling agent (e.g. a polyoxyethylene-polyoxypropylene copolymer); a preservative (e.g. benzyl alcohol, benzalkonium chloride, chlorhexidine, chlorbutol, a benzoate, potassium sorbate or EDTA or salt thereof); and pH buffering agent(s) (such as a mixture of dihydrogen

phosphate and hydrogen phosphate salts, or a mixture of citric acid and a hydrogen phosphate salt).

A pharmaceutically acceptable carrier can also be incorporated in the formulation of the present invention and can be any carrier conventionally used in the art. Examples thereof include water, lower alcohols, higher alcohols, polyhydric alcohols, monosaccharides, disaccharides, polysaccharides, hydrocarbon oils, fats and oils, waxes, fatty acids, silicone oils, nonionic surfactants, ionic surfactants, silicone surfactants, and water- based mixtures and emulsion-based mixtures of such carriers. The term "pharmaceutically acceptable" or "pharmaceutically acceptable carrier" is used herein to refer to a compound or composition that can be incorporated into a pharmaceutical formulation without causing undesirable biological effects or unwanted, interaction with other components of the formulation, "carriers" or "vehicles" as used herein refer to carrier materials suitable for incorporation in a topically applied composition. Carriers and vehicles useful herein include any such materials known in the art, which are non-toxic and do not interact with other components of the formulation in which it is contained in a deleterious manner. The term "aqueous" refers to a formulation that contains water or that becomes water-containing following application to the skin or mucosal tissue.

Cream bases are water-washable, and contain an oil phase, an emulsifier, and an aqueous phase. The oil phase, also called the "internal" phase, is generally comprised of petrolatum and a fatty alcohol such ascetyl or stearyl alcohol. The aqueous phase usually, although not necessarily, exceeds the oil phase in volume, and generally contains a humectant. The emulsifier in a cream formulation is generally a nonionic, anionic, cationic or amphoteric surfactant.

Lotions are preparations to be applied to the skin surface without friction, and are typically liquid or semiliquid preparations in which particles, including the active agent, are present in a water or alcohol base. Lotions are usually suspensions of solids, and preferably, comprise a liquid oily emulsion of the oil-in-water type. Lotions are preferred formulations herein for treating large body areas, because of the ease of applying a more fluid composition. It is generally necessary that the insoluble matter in a lotion be finely divided. Lotions will typically contain suspending agents to produce better dispersions as well as compounds useful for localizing and holding the active agent in contact with the skin, e.g., methylcellulose, sodium carboxymethyl-cellulose, or

the like. Solutions are homogeneous mixtures prepared by dissolving one or more chemical substances (solutes) in a liquid such that the molecules of the dissolved substance are dispersed among those of the solvent. The solution can contain other pharmaceutically or cosmetically acceptable chemicals to buffer, stabilize or preserve the solute. Common examples of solvents used in preparing solutions are ethanol, water, propylene glycol or any other acceptable vehicles. As is of course well known, gels are semisolid, suspension-type systems. Single-phase gels contain organic macromolecules distributed substantially uniformly throughout the carrier liquid, which is typically aqueous, but also, preferably, contain an alcohol, and, optionally, an oil. Preferred organic macromolecules, *i.e.*, gelling agents, are cross-linked acrylic acid polymers such as the "carbomer" family of polymers, e.g., carboxypolyalkylenes that can be obtained commercially under the Carbopol trademark. Also preferred are hydrophilic polymers such as polyethylene oxides, polyoxyethylene-polyoxypropylene copolymers and polyvinylalcohol; cellulosic polymers such as hydroxy-propyl cellulose, hydroxyethyl cellulose, hydroxypropyl methylcellulose, hydroxy-propyl methylcellulose phthaiate, and methylcellulose; gums such as tragacanth and xanthan gum; sodium alginate; and gelatin, In order to prepare a uniform gel, dispersing agents such as alcohol or glycerin can be added, or the gelling agent can be dispersed by trituration, mechanical mixing or stirring, or combinations thereof. Ointments, as also well known in the art, are semisolid preparations that are typically based on petrolatum or other petroleum derivatives. The specific ointment base to be used, as will be appreciated by those skilled in the art, is one that will provide for a number of desirable characteristics, *e.g.*, emolliency or the like. As with other carriers or vehicles, an ointment base should be inert, stable, nonirritating, and nonsensitizing. As explained in Remington: The Science and Practice of Pharmacy, 19th Ed. (Easton, PA: Mack Publishing Co., 1995), at pages 1399-1404, ointment bases can be grouped in four classes: oleaginous bases; emulsifiable bases; emulsion bases; and water- soluble bases. Oleaginous ointment bases include, for example, vegetable oils, fats obtained from animals, and semisolid hydrocarbons obtained from petroleum.

Emulsifiable ointment bases, also known as absorbent ointment bases, contain little or no water and include, for example, hydroxystearin sulfate, anhydrous lanolin, and hydrophilic petrolatum.

Emulsion ointment bases are either water-in-oil (W/O) emulsions or oil-in- water (O/W) emulsions, and include, for example, acetyl alcohol, glyceryl monostearate,

lanolin, and stearic acid. Preferred water-soluble ointment bases are prepared from polyethylene glycols of varying molecular weight; see Remington: The Science and Practice of Pharmacy for further information.

Pastes are semisolid dosage forms in which the active agent is suspended in a suitable base. Depending on the nature of the base, pastes are divided between fatty pastes or those made from single-phase aqueous gels. The base in a fatty paste is generally petrolatum or hydrophilic petrolatum or the like. The pastes made from single-phase aqueous gels generally incorporate carboxymethylcellulose or the like as a base.

Enhancers are lipophilic co-enhancers typically referred to as "plasticizing" enhancers, *i.e.*, enhancers that have a molecular weight in the range of about 150 to 1000, an aqueous solubility of less than about 1 wt.%, preferably less than about 0.5 wt.%, and most preferably less than about 0.2 wt.%. The Hildebrand solubility parameter δ of plasticizing enhancers is in the range of about 2.5 to about 10, preferably in the range of about 5 to about 10. Preferred lipophilic enhancers are fatty esters, fatty alcohols, and fatty ethers. Examples of specific and most preferred fatty acid esters include methyl laurate, ethyl oleate, propylene glycol nionolaurace, propylene glycerol dilaurate, glycerol monolaurate, glycerol monooleate, isopropyl n-decanoate, and octyldodecyl myristate. Fatty alcohols include, for example, stearyl alcohol and oleyl alcohol, while fatty ethers include compounds wherein a diol or triol, preferably a C_2-C_4 alkane diol or triol, are substituted with one or two fatty ether substituents. Additional permeation enhancers will be known to those of ordinary skill in the art of topical drag delivery, and/or are described in the pertinent texts and literature. See, *e.g.*, Percutaneous Penetration Enhancers, eds. Smith *et al.* (CRC Press, 1995)(incorporated herein by reference herein in its entirety).

Various other additives can be included in the compositions of the present invention in addition to those identified above. These include, but are not limited to, antioxidants, astringents, perfumes, preservatives, emollients, pigments, dyes, humectants, propellants, and sunscreen agents, as well as other classes of materials whose presence can be pharmaceutically or otherwise desirable. Typical examples of optional additives for inclusion in the formulations of the present invention are as follows: preservatives such as sorbate; solvents such as isopropanol and propylene glycol; astringents such as menthol and ethanol; emollients such as polyalkylene methyl glucosides; humectants such as glycerine; emulsifiers such as glycerol stearate, PEG-

100 stearate, polyglyceryl-3 hydroxylaury 1 ether, and polysorbate 60; sorbitol and other polyhydroxyalcohols such as polyethylene glycol; sunscreen agents such as octyl methoxyl cinnamate (available commercially as Parsol MCX) and butyl methoxy benzoylmethane (available under the tradename Parsol 1789); antioxidants such as ascorbic acid (vitamin C), a-tocopherol (Vitamin E), β-tocopherol, γ-tocopherol, δ-tocopherol, ε-tocopherol, ζι -tocopherol, Z$^{\Lambda}$- tocopherol, η -tocopherol, and retinol (vitamin A); essential oils, ceramides, essential fatty acids, mineral oils, vegetable oils (*e.g.*, soya bean oil, palm oil, liquid fraction of shea butter, sunflower oil), animal oils (*e.g.*, perhydrosqualene), synthetic oils, silicone oils or waxes (*e.g.*, cyclomethicone and dimethicone), fluorinated oils (generally perfluoropolyethers), fatty alcohols (*e.g.*, cetyl alcohol), and waxes (*e.g.*, beeswax, carnauba wax, and paraffin wax); skin-feel modifiers; and thickeners and structurants such as swelling clays and cross-linked carboxypolyalkylenes that can be obtained commercially under the Carbopol trademark. Other additives include beneficial agents such as those materials that condition the skin (particularly, the upper layers of the skin in the stratum corneum) and keep it soft by retarding the decrease of its water content and/or protect the skin. Such conditioners and moisturizing agents include, by way of example, pyrrolidine carboxylic acid and amino acids; organic antimicrobial agents such as 2,4,4'- trichloro-2 -hydroxy diphenyl ether (triclosan) and benzoic acid; anti- inflammatory agents such as acetylsalicylic acid and glycyrrhetinic acid; anti-seborrhoeic agents such as retinoic acid; vasodilators such as nicotinic acid; inhibitors of melanogenesis such as kojic acid; and mixtures thereof. Further additional active agents including, for example, alpha hydroxyacids, alpha ketoacids, polymeric hydroxyacids, moisturizers, collagen, marine extract, and antioxidants such as ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), β-tocopherol, γ-tocopherol, 6- tocopherol, ε-tocopherol, ζι -tocopherol, ζ_2- tocopherol, η -tocopherol, and retinol (Vitamin A), and/or pharmaceutically acceptable salts, esters, amides, or other derivatives thereof. A preferred tocopherol compound is α-tocopherol. Additional agents include those that are capable of improving oxygen supply in skin tissue, as described, for example, in Gross, *et al.*, WO 94/00098 and Gross, *et al.*, WO 94/00109, both assigned to Lancaster Group AG (incorporated herein by reference in their entirety). Sunscreens and UV absorbing compounds can also be included. Non-limiting examples of such sunscreens and UV absorbing compounds include aminobenzoic acid (PABA), avobenzone, cinoxate, dioxybenzone, homosalate, menthyl anthranilate,

oxtocrylene, octyl methoxycmnamate, octyl salicylate, oxybenzone, padirnate O, phenylbenzirmdazole sulfonic acid, sulisobenzone, titanium dioxide, trolamine salicylate, zinc oxide, ensulizole, meradiraate, octinoxate, octisalate, and octocrylene. See Title 21. Chapter 1. Subchapter D. Part 352. "Sunscreen drug products for over-the-counter human use" incorporated herein in its entirety. Other embodiments can include a variety of non-carcinogenic, non-irritating healing materials that facilitate treatment with the formulations of the invention. Such healing materials can include nutrients, minerals, vitamins, electrolytes, enzymes, herbs, plant extracts, glandular or animal extracts, or safe therapeutic agents that can be added to the formulation to facilitate the healing of dermal disorders.

The present invention contemplates amounts of these various additives equivalent to those conventionally used in the cosmetics field, and range, for example, from about 0.01 % to about 20% of the total weight of the topical formulation.

The formulations of the invention can also include conventional additives such as opacifiers, fragrance, colorant, stabilizers, surfactants, and the like. In certain embodiments, other agents can also be added, such as antimicrobial agents, to prevent spoilage upon storage, *i.e.*, to inhibit growth of microbes such as yeasts and molds.

Suitable antimicrobial agents for the present invention include, but are not limited to the following selected from the group consisting of the methyl and propyl esters of p-hydroxybenzoic acid (i.e., methyl and propyl paraben), sodium benzoate, sorbic acid, imidurea, and combinations thereof. In other embodiments, other agents can also be added, such as repressors and inducers, i.e., to inhibit (*i.e.*, glycose) or induce (i.e. xylose) the production of the polypeptide of interest. Such additives can be employed provided they are compatible with and do not interfere with the function of the formulations.

The formulations can also contain irritation-mitigating additives to minimize or eliminate the possibility of skin irritation or skin damage resulting from the chemical entity to be administered, or other components of the composition.

Suitable irritation-mitigating additives include, for example: α-tocopherol; monoamine oxidase inhibitors, particularly phenyl alcohols such as 2-phenyl- 1 -ethanol; glycerin; salicylates; ascorbates; ionophores such as monensin; amphophilic amines; ammonium chloride; N-acetylcysteine; capsaicin; and chloroquine. The irritation-mitigating additive, if present, can be incorporated into the compositions at a

concentration effective to mitigate irritation or skin damage, typically representing not more than about 20 wt.%, more typically not more than about 5 wt.%, of the formulation. Further suitable pharmacologically active agents that can be incorporated into the present formulations in certain embodiments and thus topically applied along with the active agent include, but are not limited to, the following: agents that improve or eradicate pigmented or non-pigmented age spots, keratoses, and wrinkles; antimicrobial agents; antibacterial agents; antipruritic and antixerotic agents; anti-inflammatory agents; local anesthetics and analgesics; corticosteroids; retinoids; vitamins; hormones; and antimetabolites. Some examples of topical pharmacologically active agents include acyclovir, amphotericins, chlorhexidine, clotrimazole, ketoconazole, econazole, miconazole, metronidazole, minocycline, nystatin, neomycin, kanamycin, phenytoin, para-amino benzoic acid esters, octyl methoxycmnamate, octyl salicylate, oxybenzone, dioxybenzone, tocopherol, tocopheryl acetate, selenium sulfide, zinc pyrithione, diphenhydramine, pramoxine, lidocaine, procaine, erythromycin, tetracycline, clindamycin, crotamiton, hydroquinone and its monomethyl and benzyl ethers, naproxen, ibuprofen, cromolyn, retinol, retinyl palmitate, retinyl acetate, coal tar, griseofulvin, estradiol, hydrocortisone, hydrocortisone 21-acetate, hydrocortisone 17-valerate, hydrocortisone 17-butyrate, progesterone, betamethasone valerate, betamethasone dipropionate, triamcinolone acetonide, fluocinonide, clobetasol propionate, minoxidil, dipyridamole, diphenylhydantoin, benzoyl peroxide, and 5-fluorouracil. A cream, lotion, gel, ointment, paste or the like can be spread on the affected surface and gently rubbed in. A solution can be applied in the same way, but more typically will be applied with a dropper, swab, or the like, and carefully applied to the affected areas.

The application regimen will depend on a number of factors that can readily be determined, such as the severity of the condition and its responsiveness to initial treatment, but will normally not involve more than one application per day. One of ordinary skill can readily determine the optimum amount of the formulation to be administered, administration methodologies and repetition rates. In general, it is contemplated that the formulations of the invention will be applied in the range of once or twice weekly up to once daily.

METHODS

Methods of Treatment

The invention provides methods for treating a skin disease, wherein the methods comprise administering to a subject in need of such treatment a genetically engineered microorganism, *e.g.*, genetically engineered bacteria, capable of expressing a recombinant therapeutic fusion protein of the invention, thereby treating the subject. In some aspects, the invention provides methods for treating a skin disease, wherein the methods comprise administering to a subject in need of such treatment a pharmaceutical composition comprising a filaggrin polypeptide described herein. In some embodiments, the filaggrin polypeptide is a fusion protein. In a further embodiment, the filaggrin polypeptide comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1.

In one embodiment, the disease is atopic dermatitis.

In one embodiment, the disease is Ichthyosis vulgaris (IV). Clinical features of IV usually appear at around 2 months of age and include generalized xerosis and fine, white to gray scales that are prominent on the abdomen, chest, and extensor surfaces of the extremities. IV is commonly caused by a genetic mutation that's inherited from one or both parents. The scales of ichthyosis vulgaris, sometimes called fish scale disease or fish skin disease, can be present at birth, but usually first appear during early childhood. In one embodiment, the subject to be treated is a child. Sometimes other skin diseases, such as the allergic skin condition eczema, are associated with ichthyosis vulgaris.

In yet another preferred embodiment, the recombinant therapeutic fusion protein comprises filaggrin. In other embodiments, the recombinant therapeutic fusion protein comprises filaggrin operably linked to a cell penetrating peptide. In one embodiment, the filaggrin fusion protein comprises an amino acid sequence that is 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO. 1.

In further embodiments, the recombinant therapeutic fusion protein is operably linked to an export signal.

KITS

The present invention also provides kits. In one aspect, a kit of the invention comprises (a) a composition of the invention and (b) instructions for use thereof. In

another aspect, a kit of the invention comprises (a) any one of the live biotherapeutic compositions of the invention, and (b) instructions for use thereof. The compositions of the invention are described *supra*. In some embodiments, a composition of the invention is an engineered microorganism capable of expressing therapeutically relevant recombinant fusion polypeptides, as described *supra*. In preferred embodiments, the composition comprises engineered bacteria (*e.g.*, *S. epidermidis*) capable of expressing a recombinant fusion polypeptide comprising filaggrin.

The present invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all figures and all references, patents and published patent applications cited throughout this application, as well as the Figures, are expressly incorporated herein by reference in their entirety.

EXAMPLES

The following examples further describe and demonstrate embodiments within the scope of the present invention. The Examples are given solely for purpose of illustration and are not to be construed as limitations of the present invention, as many variations thereof are possible without departing from the spirit and scope of the invention.

Example 1. Quantification and Comparison of Growth Characteristics of Transformed Bacteria in Liquid Media.

It was important to gain a basic understanding of the ability of transformed *Staphylococcus epidermidis* (SE) to compete against wild type SE. In order to understand the growth characteristics of the transformed bacteria and the growth dynamics of recombinant, protein-producing bacteria, standard methods of growth in liquid media were used. In order to determine growth differences between SE-sGFP, SE-chl (empty vector control, resistant to chloramphenicol (chl)), and wild type SE, each strain was grown separately in 100 mL cultures each for 6 hours. Every 45 minutes (the approximate doubling time of SE), 1 mL samples were taken and measured at 600 nm to obtain measurements of the total concentration of bacteria. Optical density was compared across all samples to understand growth characteristics.

Results are shown in FIG. 1. As shown in FIG. 1, protein production only slightly diminished the competitive growth of SE-GFP relative to SE as determined by CFU measurements. It is expected that diminished growth is due to the increased metabolic demand in protein-producing SE.

Example 2. Quantification of Growth of *SE-GFP* and Control Strains on Reconstituted Human Epidermis (RHE).

In order to characterize the feasibility of applying bacteria to the skin, the growth dynamics of externally applied bacteria on an *in vitro* skin model were determined. This model provided an initial approximation of the ecological competition these bacteria would encounter on human skin. RHE cultures were established and maintained in antibiotic- and antifungal-free media (supplemented with chloramphenicol as needed). *SE-sGFP* suspended in 50% glycerol were applied with a pipette to the center 3mm diameter of the RHE. Control RHE with *SE-chl* and *SE-WT* bacteria were also applied alongside the experimental arms. At pre-determined timepoints, the tissue inserts removed, homogenized and passed through a 40μm filter to allow for collection of bacterial flow through. The bacterial suspension was spun down, resuspended in media, serially diluted and plated to determine the CFUs of bacteria. All measurements were normalized to the maximum recovery of bacteria as determined by the CFUs present 15 minutes after application.

Results are shown in FIG. 2. As shown in FIG. 2, *SE-GFP* grew more slowly than *SE* with an empty vector when applied on RHE separately. While growth of control *SE* plateaus by 24 hours, *SE-GFP* bacteria plateau by 48 hours and the final counts are ~ ½ log lower. When added together to simulate the competition on human skin, *SE* with the vector alone outgrew and slightly outcompeted the protein-producing *SE* with at least one log difference of CFU (FIG. 2). These results, combined with those above, led to the conclusion that protein-producing *SE* may have a slight disadvantage when colonizing the skin in a clinical setting, potentially requiring additional genetic modification to facilitate growth and competition. However, as shown in Example 3, these bacteria are well able to colonize skin models.

Example 3. Qualitative Characterization of Growth of *SE-GFP* and Control Strains on RHE.

The purpose of this experiment was to add spatial and temporal information about *SE-GFP* colonization using RHE and the Vivascope. *SE-GFP* was applied to RHE for 3.5 hours, and samples were imaged in reflectance and fluorescence modes in three standardized regions of 2 mm x 2 mm wide and 100 µm deep using 4.75 µm steps and linear increase in laser power. Ultrasound gel (Parker Laboratories) was used to preserve the refractive index between the objective and the glass sample plate. Importantly, in order to mimic the hyperstructure of damaged skin in AD patients, the RHE was intentionally punctured with a Derma Microneedle device to determine the localization of the bacteria in the presence of damaged skin. The results showed that the bacteria, applied at 10^8 CFUs and grown at 3.5 hours, localized to the puncture at depths up to 70 µm (arrows, FIG. 3B and FIG. 3C). This suggests that the topically applied bacteria will be able to hone to areas of damaged skin.

The results from these experiments showed that the modified bacteria hone to the surface and deep grooves of the stratum corneum layer and maintain a constant presence over the course of the experiment. These experiments also showed that *SE* hone to skin barrier defects and this suggests that our bacteria would colonize more deeply in skin that is impaired, as we expect in AD patients (FIG. 3A-C).

Example 4. Characterization of Delivery of Bacterially Secreted sGFP to the Skin Using an *in vitro* Model System.

Isolation of sGFP produced by SE

Protein was purified from 225 mL culture of *SE* (2.7-2.9 OD/mL and lysed with CellB-Lyse (Sigma). 4 mL eluate was produced and ran with 500 mM imidazole from a Ni-NTA column in a BPS buffer at pH 7.4 and yielded 0.4 mg per 225 mL of SE. SDS-PAGE gels of purified sGFP are shown in FIG. 4.

Characterize delivery of bulk purified sGFP and sGFP+RMR to RHE and characterize cellular compartment and depth of penetration sGFP protein from SE-GFP reporter and control strains.

The localization of purified sGFP and sGFP+RMR was examined to answer three important questions: (1) does sGFP+RMR penetrate the stratum corneum; (2) if so, how deeply can it be detected; and; (3) what are the kinetics of penetration.

5.0 μg of GFP +/- RMR was applied to RHE. Two-photon microscopy and confocal imaging were used to examiner the effects at 30 minutes and 60 minutes to examine penetration and kinetic. Importantly, both intracellular localization of GFP due to the effects of RMR to penetrate human cells was examined, as well as the transdermal localization of GFP due to the effects of RMR in facilitating dermal penetration.

The results of these experiments showed that GFP without the RMR did not effectively penetrate RHE (FIG. 5A, B, E, F). However, the same amount of GFP-RMR was more deeply delivered into the tissue (FIG. 5G, H, J) compared to GFP without the RMR signal (FIG. 5E, F, K), supporting the efficacy and necessity of the RMR for transdermal penetration. Additionally, evidence of GFP with the RMR accumulating in keratinocytes was found (FIG. 5C, D), compared to GFP without the tag (FIG. 5 A, B), supporting intracellular penetration. Overall, these results indicate that the RMR facilitate transdermal delivery of our protein, which is highly relevant for delivering filaggrin more deeply into the skin of IV patients with treatment with filaggrin-producing *SE*. Depth and localization of GFP following breaching the stratum corneum with the Dermaroller was examined, and it was found that the GFP preferentially localized and penetrated into the breached area (FIG. 5O).

Example 5. Analytical Method Development and Optimization of Human Filaggrin Production from *Staphylococcus epidermidis.*

Analytical methods for filaggrin quantification were developed, as well as a structure-activity approach to rationally select the optimal filaggrin oligomer and sequence to move forward in preclinical and subsequently clinical development.

Rational Design of Filaggrin Sequence

A filaggrin molecule that was based on what is thought to be a domain containing sequences flanked by proposed linker regions at both ends was used as the starting point. hFLG is comprised of 12 repeating units and is processed by intracellular proteases to release the individual units. An overview of filaggrin structure is presented in FIG. 6E. See FIG. 6A, B for bioinformatics analyses studying the homology and

sequence identify of the human filaggrin domains. The sequences within these units are highly homologous. It has been proposed that the units are cleaved at "linker regions." The segments between these linker regions are the individual units, with each unit containing a pair of sub-domains. The spatial orientation or topography of these units has not been resolved because the molecules are not highly structurally organized. Based on Kyte-Doolittle hydropathy scoring, the linker regions were defined as the most lipophilic amino acid segments in the hFLG protein (FIG. 6C, D).

In an effort to study the SAR of hFLG, the entire unit containing sub-domains 9 and 10 flanked by their linker regions were recombinantly produced. To identify the minimum protein segment responsible for keratin binding activity, therefore, proteins were produced with systematically shaved off regions of the N-terminus. The role of the linker region is also examined. To address this question, proteins are being prepared where the linker region is centered within the sequence. An RMR peptide sequence is shown in FIG. 6E, FIG. 6E based on the previous characterization of the delivery of GFP via an RMR (FIG. 5A-N). As described below, a structure-activity relationship (SAR)-based approach to select the optimal filaggrin sequence of the possible sequences that have been identified is used.

Example 6. Identification and Quantification of Filaggrin Secreted by SE.

Critical to later preclinical and clinical development of a filaggrin-secreting *SE* strain, methods to identify and quantify filaggrin secreted by *SE* were developed.

Antibody development and immunoreactive detection of hFLG

An antibody (RL-012-001B, a chicken IgY), was developed against an epitope of hFLG that is common across all pairs of sub-domains. This was necessary because commercially available filaggrin antibodies displayed significant non-specific binding. This IgY antibody demonstrates a clean band on Western blots for filaggrin isolated from cells lysate of filaggrin-producing *SE* and its media (FIG. 7D). The IgY was able to recognize several different pairs of hFLG subunits that were produced (FIG. 7 B-D).

Mass spectrometry (MS)

Preliminary LC-DDA- (Data Dependent Acquisition) MS/MS analysis of 20 ng of human filaggrin segments 9-10 are presented in FIG. 7E. This identifies peptide

sequences that can be followed as filaggrin production, secretion and delivery is monitored.

The protein sample is re-suspended in 50 mM ammonium bicarbonate buffer and digested with trypsin by incubating overnight at 37°C. The sample is then acidified to halt the trypsin activity, and then desalted using C18 spin columns, the eluates of which are then dried under vacuum-centrifugation and re-suspended in 0.1% formic acid. The resulting trypsin digest is analyzed by reverse-phase LC-DDA-MS/MS using nanoACQUITY UPLC system connected to an Orbitrap-Fusion Tribrid mass spectrometer equipped with a nanoelectrospray source. After injection, the peptides are loaded into a trapping column and then separated with a C18 column.

LC-DDA-MS/MS analysis is performed at with the mass spectrometer operating in a Top Speed data-dependent mode with maximum total cycle time of 3 sec. During MS1 full scan, precursor ions are isolated in the quadrupole mass analyzer, recorded with a maximum injection time of 60 ms, an automatic gain control (AGC) target value of 4×10^5, and an Orbitrap resolution of 120,000 at *m/z* 200. A full scan is acquired in the range of 300-1500 m/z, followed by MS2 of the most intense ions. The ions are iteratively isolated with a 1.6 Th window, injected with a maximum injection time of 110 ms, AGC target of 1×10^5, and then fragmented with higher-energy collisional dissociation (HCD) of 28. The MS/MS spectra are then recorded at a resolution of 60,000 at *m/z* 200. Ions with single charge or unassigned charge states are excluded from fragmentation.

The reliability and robustness of this assay are critically important for biochemistry, manufacturing, and controls of any eventual LBP comprised of filaggrin-secreting *SE*. Qualification of the MS analytical methods uses the following metrics: (1) specificity; (2) sensitivity (Limit of Detection (LOD) and Limit of Quantitation (LOQ); and (3) Linearity. This will be accomplished using reference standards of purified hFLG and running multiple samples over a range of concentrations. As illustrated in FIG 7E, specific peptides are detectable, and several of these can be prepared synthetically as reference standards, if necessary.

Example 7. Keratin Binding Assay to Measure Activity of Filaggrin.

A keratin binding assay is used to measure activity of filaggrin. This will be assessed by surface plasmon resonance (SPR) method (Biacore GE Healthcare) to rank

different filaggrin segment based on their cytokeratin interaction. This will measure the binding affinity, specificity, concentration, and kinetics to examine filaggrin-cytokeratin binding dynamics. Expected metrics produced by this method include K_{on}, K_{off} and K_d. Different human cytokeratins will be evaluated starting with cytokeratin 1 and 10, known to interact with hFLG. The assay will be developed with our current filaggrin 9-10 construct and will be used to measure addition filaggrin sequences to guide the SAR analysis. The assay workflow is presented in FIG. 8.

Example 8. Optimization of Human Filaggrin Through Structure-Activity Relationship (SAR)

Based on the methods described above and shown in FIG. 6, the remaining 11 filaggrin domains will be tested and a matrix of identity and activity will be generated, as outlined by the above methods (i.e. Western blots, MS, and surface plasmon resonance (SPR) protein-protein interaction assays). The correlation of protein sequence and *in vitro* activity will guide the selection of the protein to be produced as a potential therapeutic agent.

Example 9. Conduct Analytical Method Development and Optimization of Human Filaggrin Delivery from *Staphylococcus epidermidis* to a Human Skin Model.

Critical to the characterization of later pharmacokinetics (PK), assays will be developed for detection and distribution of filaggrin. This will include characterization of filaggrin distribution in human skin models (reconstructed human epidermidis) as well as mouse skin from filaggrin-producing *SE* using MS (as described above) to detect intact and breakdown products, as well as immunofluorescence and immunohistochemistry. The measurement of filaggrin breakdown products will also supplement the characterization of activity of filaggrin, i.e. internationalization and processing of filaggrin. Additionally, assays will be developed to detect filaggrin breakdown products, including Raman spectroscopy.

Flg-/- Reconstructed human epidermis (RHE)

Filaggrin KO reconstructed human epidermis, branded as EpiDerm, will be purchased from MatTek (Ashland, MA). The Epiderm model is constructed from normal human epidermal keratinocytes (NHEK) from neonatal foreskin or adult breast skin of a

single donor. The model is supplied as 9mm diameter tissue samples that have been grown on Millicell CM (Millipore) exposed to an air-liquid interface. Epiderm recapitulates eight to twelve layers total of basal, spinous, and granular differentiation and ten to fifteen layers of stratum corneum. Histologically, metabolically, and genetically, Epiderm provides excellent correspondence to human skin. Models maintain morphology for up to three weeks. Filaggrin knockdown in keratinocytes will be achieved via shRNA lentiviral-based knockdown of *filaggrin*. An estimated 96 RHE samples will be needed for this aim.

Mouse models

Both wild type BALB/c and *flg-/-* mice will be used. BALB/c mice will be purchased from Jackson Laboratory, Bar Harbor, ME, and *flg-/-* have been obtained by Azitra from Riken Laboratory (Nagaski, Japan). An estimated 22 BALB/c and 22 *flg-/-* will be needed for this aim.

Immunofluorescence and immunohistochemistry

To understand and characterize the absorption and biodistribution of filaggrin secreted by the *SE,* immunofluorescence (IF) microscopy and immunohistochemistry (IHC) will be used to visualize filaggrin localization and distribution from filaggrin-producing SE. To do this, *flg-/-* reconstructed human epidermis (RHE) will be used. Briefly, a range of 10^6 to 10^8 colony forming units (CFUs) of filaggrin-producing *SE* will be applied to the RHE and mouse skin and incubated for 24 hours without antibiotics. Samples will be fixed for IF and IHC analyses at 24, 48, and 72 hours in triplicates and untreated RHE and mouse samples will serve as negative controls for these analyses.

For IF, samples will be fixed with 70% ethanol, 50 mM glycine for 1 hour. Immunofluorescence staining will be performed by incubation of our developed anti-filaggrin primary antibody (RL-012-001B) at 1:200 for 2 hours, followed by incubation with rat anti-goat rhodamine secondary antibody (Jackson Laboratory) at 1:200 dilutions in the presence of Hoechst Stain Solution (Sigma). Slides will be mounted with coverslips in Gel/Mount (Biomed).

For IHC, samples will be fixed in 10% formalin and paraffin embedded. Paraffin sections will be dewaxed and washed with 95% ethanol followed by methanol hydrogen

peroxide. The sections will then be treated with a heat induced epitope retrieval (HIER) procedure using rodent Decloaker solution (Biocare Medical, RD913) and the Biocare decloaking chamber. After being washed in Tris pH 7.4, sections will be incubated in the presence of rat serum and FcBlock (24G2) followed by rabbit anti-*S. epidermidis* diluted in the blocking solution. Samples will be washed in Tris and then incubated with goat anti-rabbit IgG-Texas Red antibody (Invitrogen, T2767). The tissue will then be counterstained with HOECSHT and imaged using a Leica DM IRBE fluorescent microscope.

Raman Spectroscopy

Raman spectroscopy is a known method to identify NMF breakdown products of filaggrin.[62] During terminal differentiation, pro-filaggrin is rapidly dephosphorylated and cleaved to yield functional filaggrin monomers, which bind to and assemble keratin intermediate filaments.[63,64] Cleavage of deiminated filaggrin monomers is catalyzed by several proteases, including caspase 14,[65] calpain 1, and bleomycin hydrolase.[66] Filaggrin is then broken into natural moisturizing factors (NMFs), specifically urocanic acid (UA) pyroglutamic acid (PGA) and citrulline (CIT).[62]

Raman spectroscopy will be used to identify filaggrin breakdown products in *flg-/-* RHE models and mouse skin. The Raman effect consists of a shift in photon energy due to inelastic collisions of photons with molecules. The Raman spectrum shows the scattering intensity as a function of the frequency difference between the incident and the scattered light; this difference is known as the "Raman shift." An initial Raman spectroscopy method has been developed to identify the breakdown products of filaggrin, NMFs. The skin (RHE) can be observed directly without sample preparation of destruction of the tissue. At this time, samples of commercial standards have been used to test the method, and the results of this are presented in FIG. 9A-D.

Raman spectroscopy is capable of detecting micro-molar concentrations when optimized. Nano-molar detection levels are possible using surface-enhanced Raman spectroscopy, in which nanoparticles of a suitable material (either gold or silver) can be placed on the skin sample. These particles undergo surface plasmon resonance, thus significantly increasing the sensitivity of the Raman analysis. This will be used to identify filaggrin breakdown from filaggrin-producing *SE* applied topically to RHE. 10^8 colony forming units (CFUs) of filaggrin-producing *SE* will be applied to the RHE and

incubated for 24 hours without antibiotics. Samples will be collected and analyzed at various timepoints using Raman spectroscopy to identify NMF breakdown products.

Mass spectrometry

To estimate and characterize metabolism of filaggrin, mass spectrometry will be used. Detection of commercial standards of the NMF molecules, urocanic acid (UA) pyroglutamic acid (PGA) and citrulline (CIT), has been be performed using an Agilent HP1200 mass spectrometer coupled with AB Sciex API4000 QTrap. Standards of UA, PGA and CIT were purchased from Sigma-Aldrich and were separated with Agilent Eclipse XDB C18 column (3.5u, 2.1x100mm) injected into the spectrometer and detected by MRM (multiple reaction monitor) and Secondary-ion mass spectrometry (SIMS). SIMS appeared to be about ten times more sensitive than MRM. The Limit of Quantization (LOQ) for UA, PCA and CIT are 0.5, 1, and 0.25 ng/mL respectively, and standard curves showed correlation coefficients of 0.99. When testing for the production of NMFs from FLG in RHE experiments, the RHE tissue samples will be homogenized and extracted with perchloric acid/acetonitrile, and the resulting solution injected. Standard curves of these results are presented in FIG. 9E-G. These preliminary data suggest that CIT will probably only be detected using MS rather than Raman spectroscopy.

These parameters will be used to measure filaggrin breakdown from filaggrin-producing SE applied topically to RHE. 10^8 CFUs of filaggrin-producing SE will be applied to the RHE and incubated for 24 hours without antibiotics. Samples will be collected and analyzed at various timepoints using MS to detect NMF breakdown products. This assay will also be qualified using (1) specificity and sensitivity; (2) accuracy and precision; (3) limits of the assay using the methods described above. The results of this will inform initial metabolism and distribution of filaggrin in two models.

Example 10. Evaluate Colonization Dynamics and Activity of Candidate Filaggrin-Secreting SE in Mice.

A genetic IV mouse model (*Flg-/-*) will be used, as well as wild type mice to assess colonization dynamics of FLG-producing *SE in vivo*.

Mouse models

In order to investigate the applicability of this approach, a genetic model mouse system will be used. The filaggrin knockout mouse (*Flg-/-*). *Flg-/-* mice are filaggrin deficient and exhibit dry, scaly skin.[19] Despite marked decreases in natural moisturizing factor levels, which are filaggrin degradation products, stratum corneum (SC) hydration and TEWL are normal in *Flg-/-* mice. Antigens penetrate the *Flg-/-* SC more efficiently, leading to enhanced responses in hapten-induced contact hypersensitivity and higher serum levels of anti-ovalbumin (OVA) IgG(1) and IgE. *Flg-/-* mice are obtained from RIKEN BioResource Research Center (RIKEN BRC, Tsukuba, Ibaraki, Japan) via a material transfer agreement. Wild type mice (BALB/c) will also be used in this experiment.

Study design

The designed filaggrin sequence from Figure 6E will be used in *SE* to *Flg-/-* and BALB/c mice. The study will be conducted for four weeks using five groups in each mouse type. Mice will be assigned into the following treatment groups: topical vehicle control (50% glycerol, 50% sterilized BHI medium), topical wild type *SE* ($1.0x10^8$ CFU/cm^2 in 50% glycerol), and three doses of filaggrin-secreting *SE constructs* (SE^{FLG}) (10^6, 10^7, and 10^8 CFU/cm^2 in 50% glycerol). Each solution will be applied to the same ear and tail on each mouse daily for seven days, and mice be assessed on days 7, 14, 30, and 60 for microbiome analyses to assess colonization dynamics and on days 7 and 14 for microscopy and histology to assess localization and macroscopic changes in the skin (e.g., any signs of adverse events such as inflammation), etc. 12 mice in each arm per mouse type will be used. An overview of the study design is shown in FIG. 10.

Example 11. Colonization dynamics and persistence after application of filaggrin-secreting *SE*

In order to understand the influence of addition of *SE* on microbial load, microbial diversity, and stability of the skin microbiome, 16S rRNA sequencing will be used to measure the changes in the microbial community. Skin microbiome samples will be collected using flocked swabs from mouse tail skin at baseline, (day 0), days 7, 14, 30, and 60. DNA will be extracted using skin-specific custom protocols developed by the Oh Laboratory at The Jackson Laboratory for Genomic Medicine. To measure

microbial load, qPCR using 16S rRNA V1-V3 primers will be used, as previously described. To examine microbial community dynamics, the V1-V3 will be amplified and sequenced using 2x300bp read chemistry on an Illumina MiSeq platform. Sequences will be analyzed to the genus level as previously described[40], and operational taxonomic units (OTUs) will be defined at 97% similarity. Custom computational tools to differentiate staphylococcal species will be used to track relative abundance of applied *SE* [Oh et al., Genome Research 2013][67] (FIG. 6).

Dysbiosis will be measured using ecological metrics and community structure analyses. First, dysbiosis will be assessed as a function of diversity using the Shannon Diversity Index, which is an ecological measure of microbial communities that considers and will be compared before and after application. Additionally, community structures of the local microbiome before and after treatment will be compared. Dysbiosis will be measured as % overall deviation from (1) the baseline microbiome, and (2) deviation from the mean community structure across our controls. Statistics such as the Yue-Clayton index that compares community structures will be used. Finally, microbiome trends will be analyzed on a per-species level.

Because it is not anticipated that the *S. epidermidis* will result in elimination of the endogenous flora, the longitudinal dynamics of each species over the treatments will be tracked, to identify if species are being lost from the community at a targeted sequencing depth of 50,000 reads/sample.

An experimental outline of the 16S sequencing is shown in FIG. 11.

Histology and safety analyses

Histology will be used to examine *SE* localization as well as an evaluation to probe safety and monitor any signs of inflammation. Excised ears of each group will be fixed in 4% paraformaldehyde for 16 h and will be embedded in paraffin. Then, 6 μm sections will be stained with hematoxylin (Sigma Aldrich, St Louis, MO, USA) and eosin (Sigma Aldrich, St Louis, MO, USA) (H&E). Infiltrated lymphocytes and spongiosis in the dermis will be observed by microscope (100X, 200X).

Statistical analyses

Differences between groups for the primary outcome, the macroscopic clinical disease score, will be assessed using two-sided student t-tests, if the data are normally

distributed. If not (for example, microbiome data), nonparametric equivalents such as the Wilcoxon-rank sum test will be used. Differences across groups will be assessed with ANOVA or nonparametric equivalents (Kruskal-Wallis). The same technique will be used for assessing the TEWL and the thickening of the epidermis. Finally, differences in ordinal variables will be assessed using Chi-square tests. All *P-values* will be corrected, if necessary, for multiple comparisons using false discovery rate.

For analyses of the microbiome, community variation among samples will be calculated using the quantitative, taxonomy-based Yue-Clayton distance. Beta diversity metrics (e.g., Yue-Clayton theta (ϑ) index), a metric that measures the similarity between two samples, accounting for shared species and their abundance will be used. Pairwise comparisons between each sample within a timeseries will create a baseline for intramouse, longitudinal variation. For each set of successive samples, the ϑ index will be calculated. The ratio between successive ϑ and baseline ϑ will then be used as a flux score to proxy instability over time. This allows stability to be scored based on sliding windows rather than an aggregate comparison of differences over time, which can be a confounder in longitudinal data. Dynamical systems models for time series data, such as generalized Lotka-Volterra non-linear differential equation[63,64] time-dependent generalized additive models[65] or non-parametric methods, such as Gaussian Process Dynamical Models[66] will be investigated. For individual species, models such as the Augmented Dickey-Fuller test[67] will be used to test species' homeostasis. This will determine which species contribute to variation over time, as it is possible that select microbes can account for the majority of temporal variation.

Statistical analyses

Unless otherwise indicated, experiments will be performed in triplicates, and means and standard deviations will be reported. For comparisons between groups, two-sided t-test or analysis of variance will be used. If data are not normally distributed, these will be replaced with non-parametric equivalents (Wilcoxon-rank sum and Kruskal-Wallis tests).

Safety and "kill switches"

A key requirement for nearly all recombinant microorganisms for clinical use is the ability to prevent undesired introduction to other individuals or environments. In

order to ensure safety of the engineered strain, the present invention, in one embodiment, uses an auxotrophic strain, which requires supplementation of key amino acids (D-ala) or a certain metabolic gene (*AlaR)* for survival, and simultaneously replaces the need for an antibiotic resistant strain for selection, the latter of which is not commercially viable. In another embodiment, the present invention integrates a "kill switch", which is based on CRISPR/Cas9 self-cleavage upon induction of a dual xylose-riboswitch promoter. In yet another embodiment, the present invention provides cell counters, which recombine out the AZT locus after a defined number of divisions, although this method would necessitate reapplication of the vehicle. To ensure the safety of the engineered *S. epidermidis* of the present invention, a CRISPR/Cas9-based kill switch, which is xylose-inducible and doubly regulated with a theophylline riboswitch, is used. The basis of this approach is that Cas9 is extremely efficient at chromosomal cleavage given a targeting guide, and since staphylococci lack canonical non-homologous end joining repair pathways, genomic cleavage results in death in the absence of a homologous recombination template. The use of a CRISPR-based system also confers great specificity, since comparative genomics can be used to design guides unique to the engineered *S. epidermidis* strain of the present invention, such that the construct is inactive if spread to other microbes by horizontal gene transfer. Finally, in one embodiment, the present invention provides a construct designed to express multiple CRISPR spacers to simultaneously target multiple genomic regions to ensure cleavage and minimize survival by reversion.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. A recombinant microorganism capable of secreting a polypeptide, wherein the recombinant microorganism comprises an expression vector comprising a first coding sequence comprising a gene capable of expressing the polypeptide and a second coding sequence comprising a gene capable of expressing a cell penetrating peptide.

2. The recombinant microorganism of claim 1, further comprising a third coding sequence comprising a gene capable of expressing an export signal.

3. The recombinant microorganism of claim 1 or 2, wherein the expression of the first coding sequence, second coding sequence and third coding sequence is under the control of a promoter.

4. The recombinant microorganism of claim 3, wherein the arrangement of the first coding sequence, second coding sequence and third coding sequences are in-frame.

5. The recombinant microorganism of claim 2, wherein the first coding sequence, second coding sequence and third coding sequence are operably linked to a promoter.

6. The recombinant microorganism of claim 2, wherein the recombinant microorganism is bacteria, or a combination of bacteria.

7. The recombinant microorganism of claim 1, wherein the polypeptide is filaggrin, or a variant thereof.

8. The recombinant microorganism of claim 1, wherein the microorganism is selected from the group consisting of Bifidobacterium, Brevibacterium, Propionibacterium, Lactococcus, Streptococcus, Staphylococcus, Lactobacillus, Enterococcus, Pediococcus, Leuconostoc, or Oenococcus, or combinations thereof.

9. The recombinant microorganism of claim 1, wherein the recombinant microorganism is *Staphylococcus epidermidis*.

10. The recombinant microorganism of claim 1, wherein the microorganism secretes a filaggrin fusion protein.

11. The recombinant microorganism of claim 10, wherein the filaggrin fusion protein comprises an amino acid sequence that is 95% identical to SEQ ID NO. 1.

12. A method for producing a live biotherapeutic composition, the method comprising:

(a) transfecting a cell with (i) a first coding sequence comprising a nucleic acid sequence capable of expressing a therapeutic polypeptide, and (ii) a second coding sequence comprising a nucleic acid sequence capable of expressing a cell penetrating peptide; and

(b) allowing the transfected cell to produce a therapeutic polypeptide fusion protein; and

(c) obtaining the live biotherapeutic composition.

13. The method of claim 12, further comprising (iii) transfecting the cell with a third coding sequence comprising a nucleic acid sequence capable of expressing an export signal.

14. The method of claim 12, wherein the first coding sequence, second coding sequence and third coding sequences are arranged in a single plasmid.

15. The method of claim 13, wherein the arrangement of the first coding sequence, second coding sequence and third coding sequences are operably linked to a promoter.

16. The method of claim 12, wherein the cell is selected from the group consisting of wherein the microorganism is selected from the group consisting of Bifidobacterium, Brevibacterium, Propionibacterium, Lactococcus, Streptococcus, Staphylococcus, Lactobacillus, Enterococcus, Pediococcus, Leuconostoc, or Oenococcus, or combinations thereof.

17. The method of claim 12, wherein the cell is Staphylococcus epidermidis.

18. The method of claim 12, wherein the therapeutic polypeptide fusion protein is a filaggrin fusion protein, or a variant thereof.

19. The method of claim 11, wherein the filaggrin fusion protein comprises an amino acid sequence that is 95% identical to SEQ ID NO. 1.

20. A composition obtained by the method of any one of claims 12 - 19.

21. The composition of claim 20, comprising a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous solution, an emulsion, a cream, a lotion, a gel, or an ointment.

22. A live biotherapeutic composition comprising a recombinant microorganism wherein the recombinant microorganism comprises

(i) a first coding sequence comprising a nucleic acid sequence capable of expressing a therapeutic polypeptide;

(ii) a second coding sequence comprising a nucleic acid sequence capable of expressing a cell penetrating peptide;

(iii) a third coding sequence comprising a nucleic acid sequence capable of expressing an export signal; and

(iiv) a promoter operably linked to the first coding sequence, the second coding sequence and the third coding sequence;

wherein the first coding sequence, second coding sequence and first coding sequence is capable of expressing a filaggrin fusion product, or variant thereof.

23. The composition of claim 22, wherein the recombinant microorganism is Staphylococcus epidermidis.

24. The composition of claim 22 or 23, wherein the export signal exports the filaggrin fusion product, or variant thereof, out of the recombinant microorganism.

25. The composition of claim 24, wherein the filaggrin fusion protein comprises an amino acid sequence that is 95% identical to SEQ ID NO. 1.

26. The composition of claim 20, wherein the cell penetrating peptide facilitates the entry of the filaggrin fusion product, or variant thereof, into a human keratinocyte.

27. The composition of claim 22, comprising a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous solution, an emulsion, a cream, a lotion, a gel, or an ointment.

28. A kit comprising the composition of any one of claims 22-27 and instructions for use.

29. A method of treating a skin disease comprising administering to a subject in need thereof the composition of any one of claims 1-10 or 18-23.

30. The method of claim 29, wherein the skin disease is Ichthyosis vulgaris (IV).

31. The method of claim 29, wherein the skin disease is atopic dermatitis.

32. A composition comprising a filaggrin polypeptide, or a variant thereof.

33. The composition of claim 32, wherein the filaggrin polypeptide is a fusion protein.

34. The composition of claim 33, wherein the filaggrin fusion protein comprises an amino acid sequence that is 95% identical to SEQ ID NO. 1.

35. The composition of claim 33, wherein the filaggrin fusion protein consists of SEQ ID NO: 1.

FIG. 1

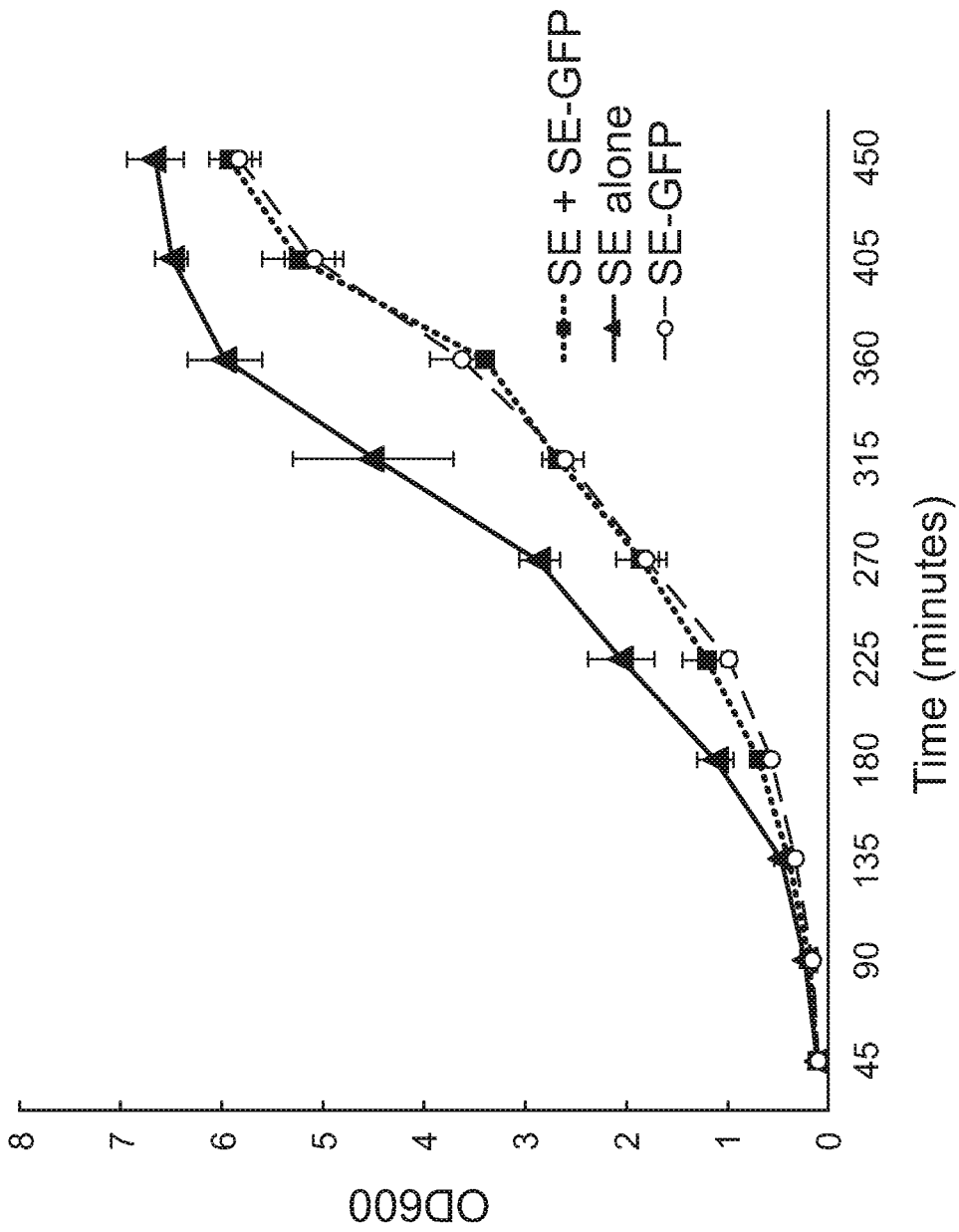

SE + SE-GFP
SE alone
SE-GFP
OD600
0
1
2
3
4
5
6
7
8
45
90
135
180
225
270
315
360
405
450
Time (minutes)

FIG. 2A

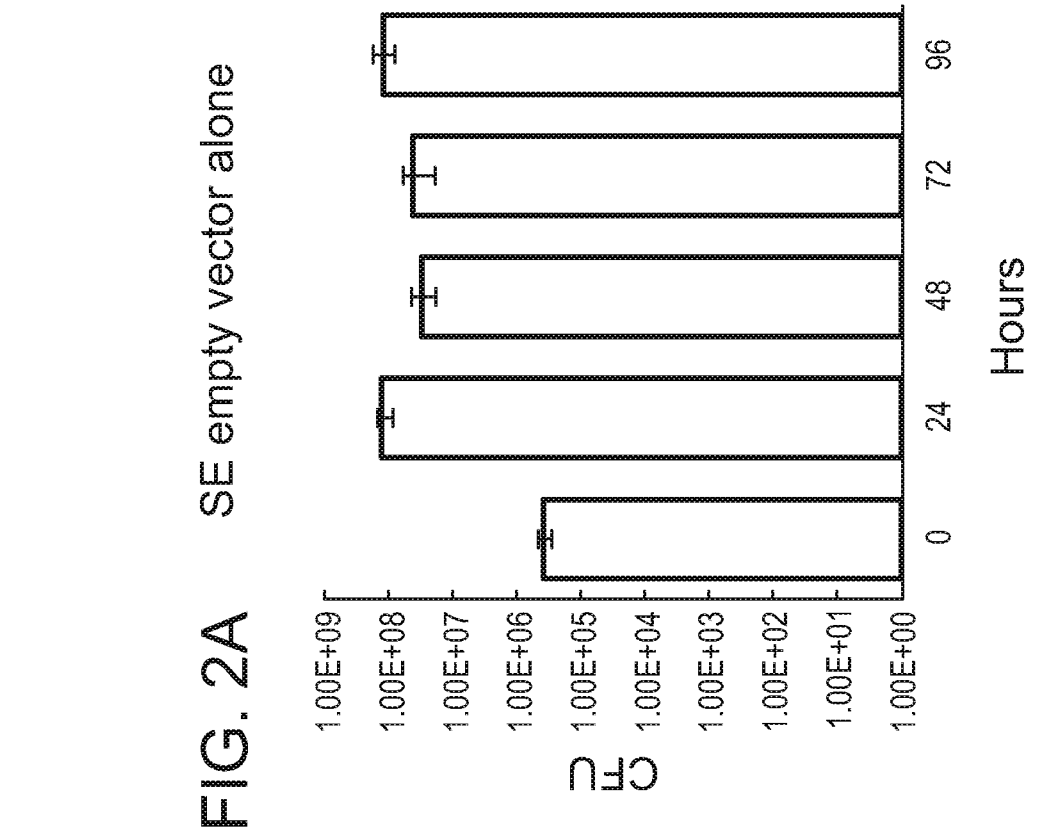
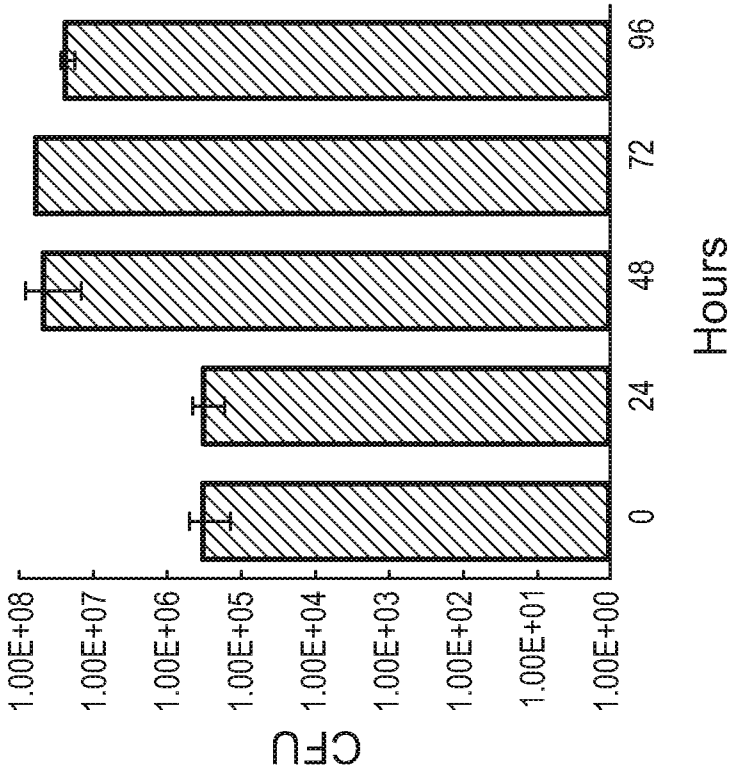

SE empty vector alone
CFU
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
24
48
72
96
Hours

FIG. 2B


SE-GFP alone
CFU
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
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1.00E+01
1.00E+00
0
24
48
72
96
Hours

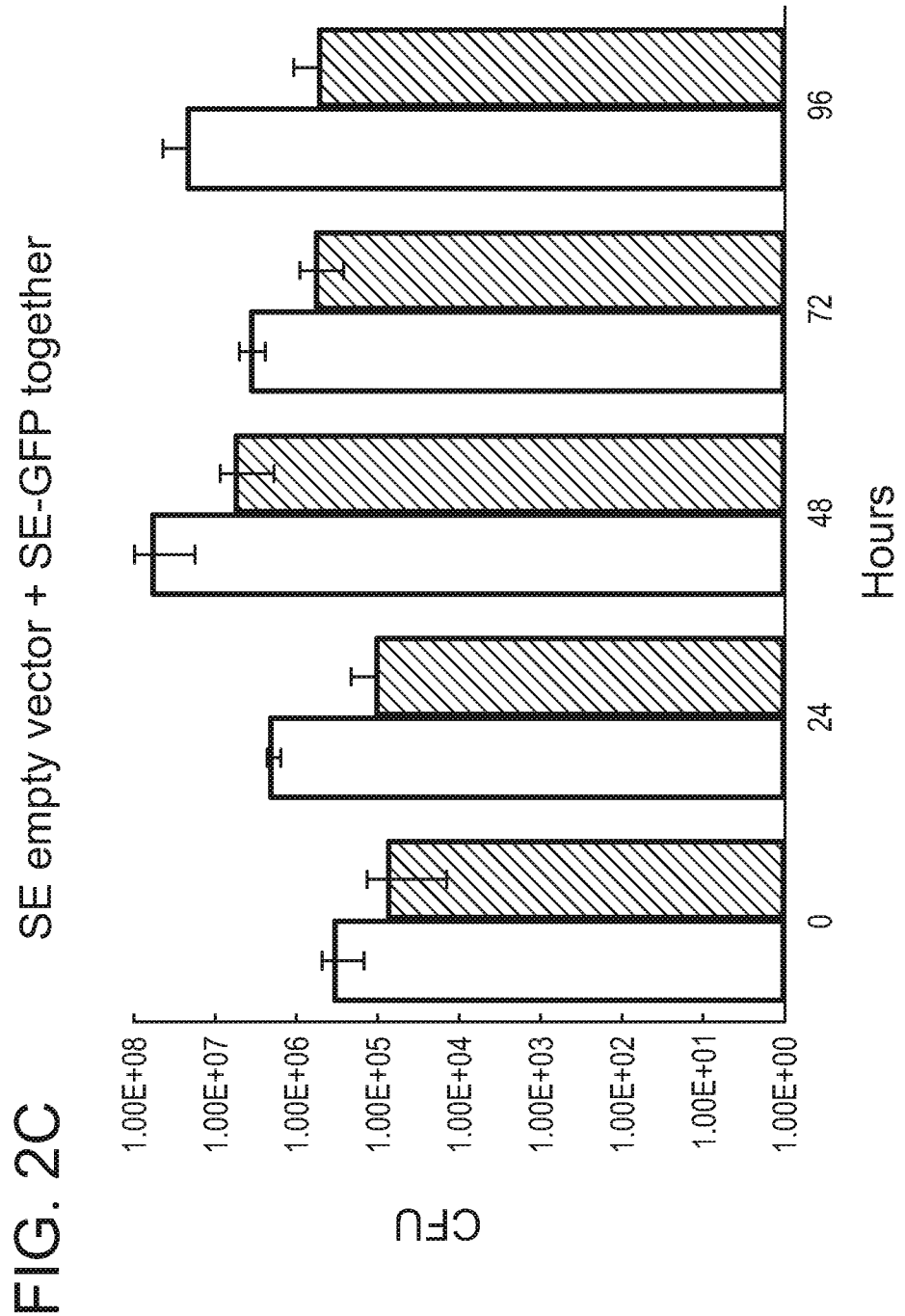

SE empty vector + SE-GFP together
CFU
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
0
24
48
72
96
Hours

FIG. 2C

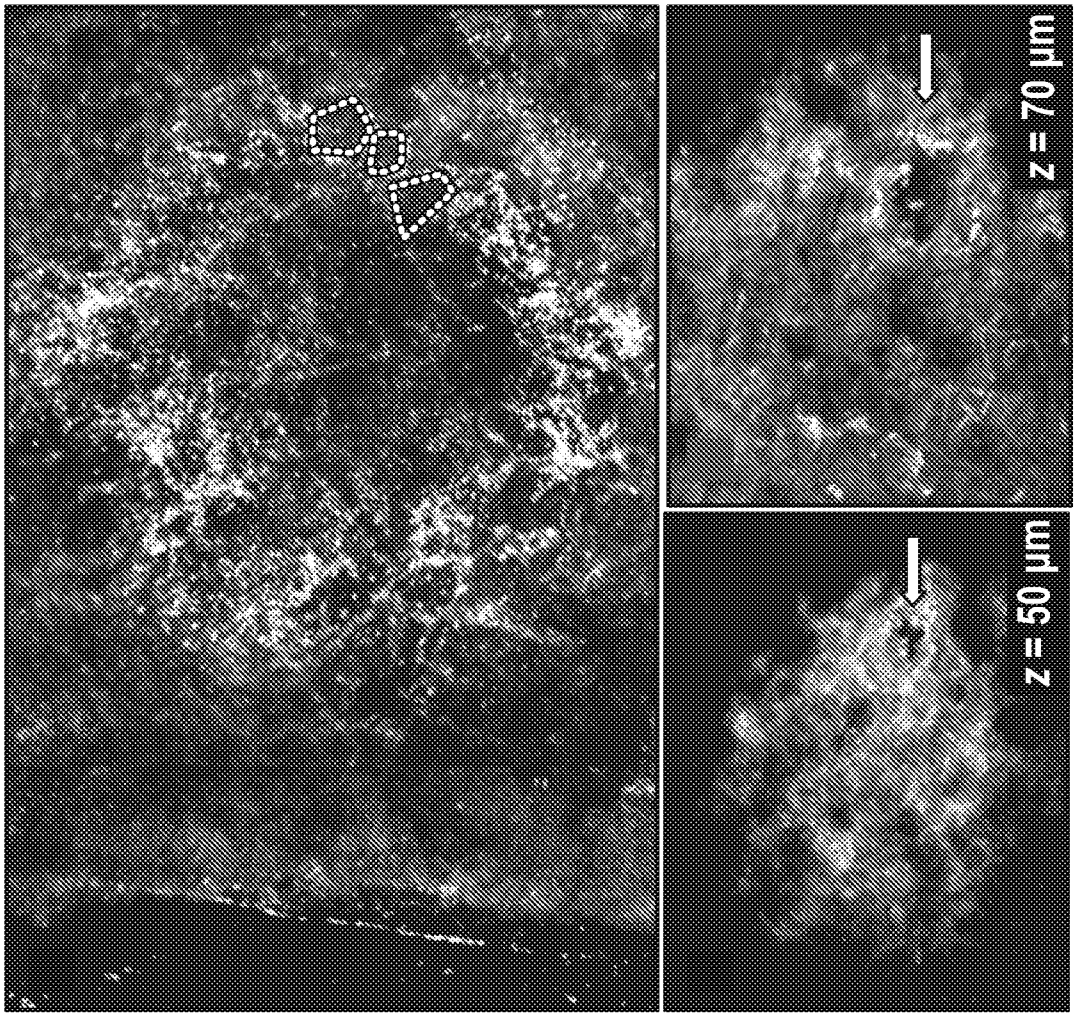


FIG. 3A

FIG. 3C

FIG. 3B

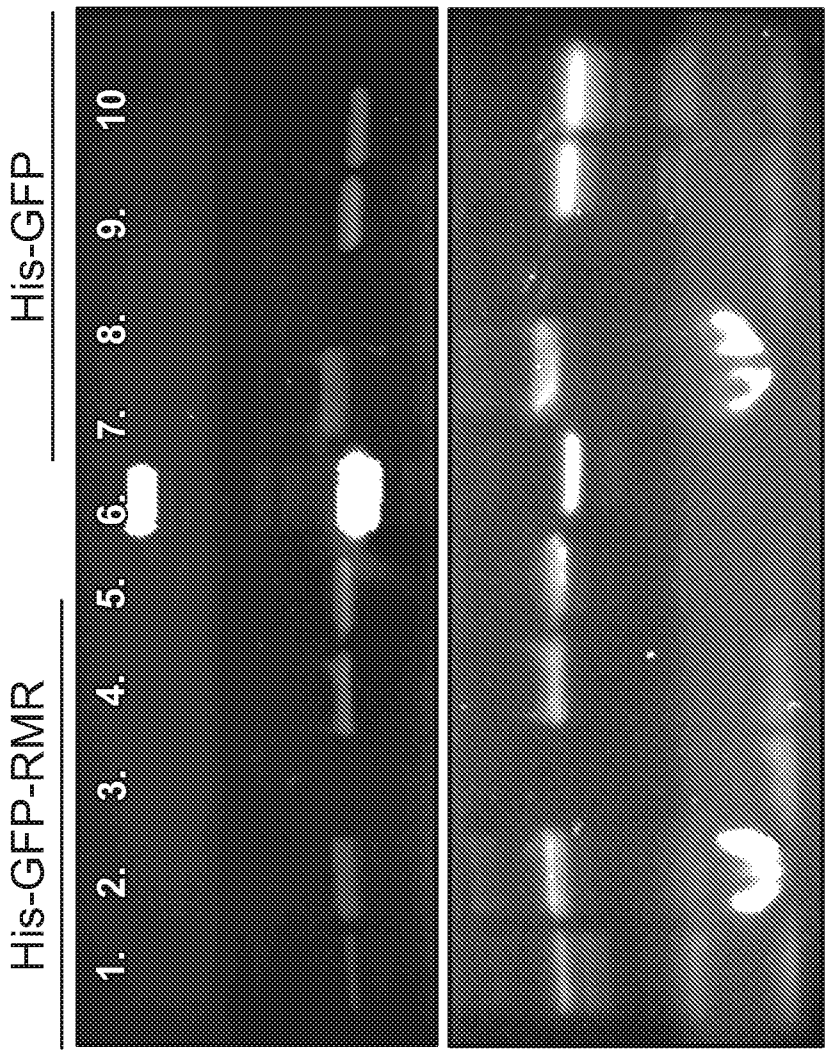


FIG. 4A

FIG. 4B

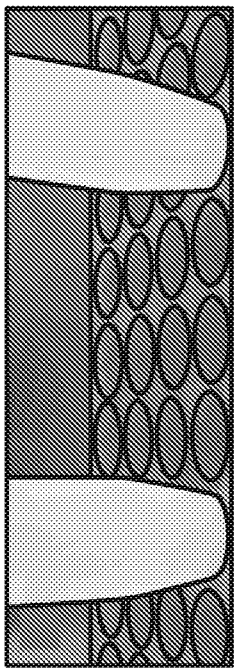


FIG. 4C

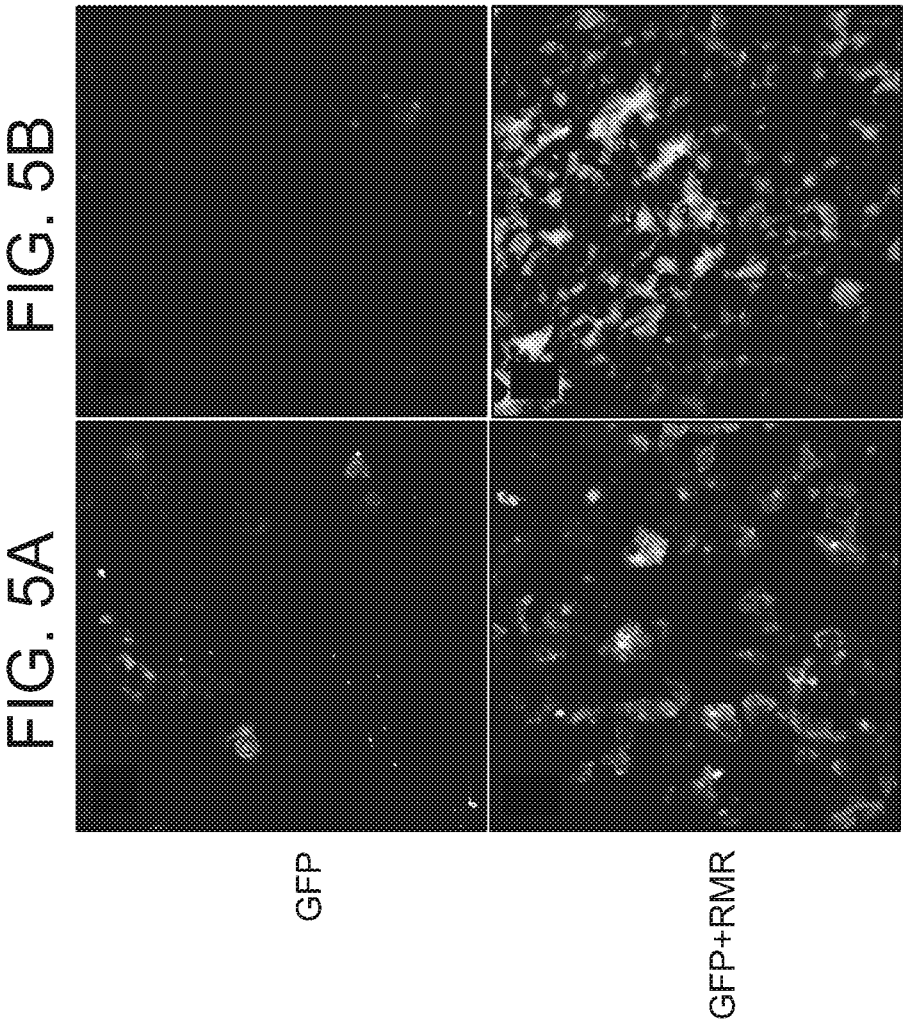


FIG. 5A
FIG. 5B
FIG. 5C
FIG. 5D
GFP
GFP+RMR

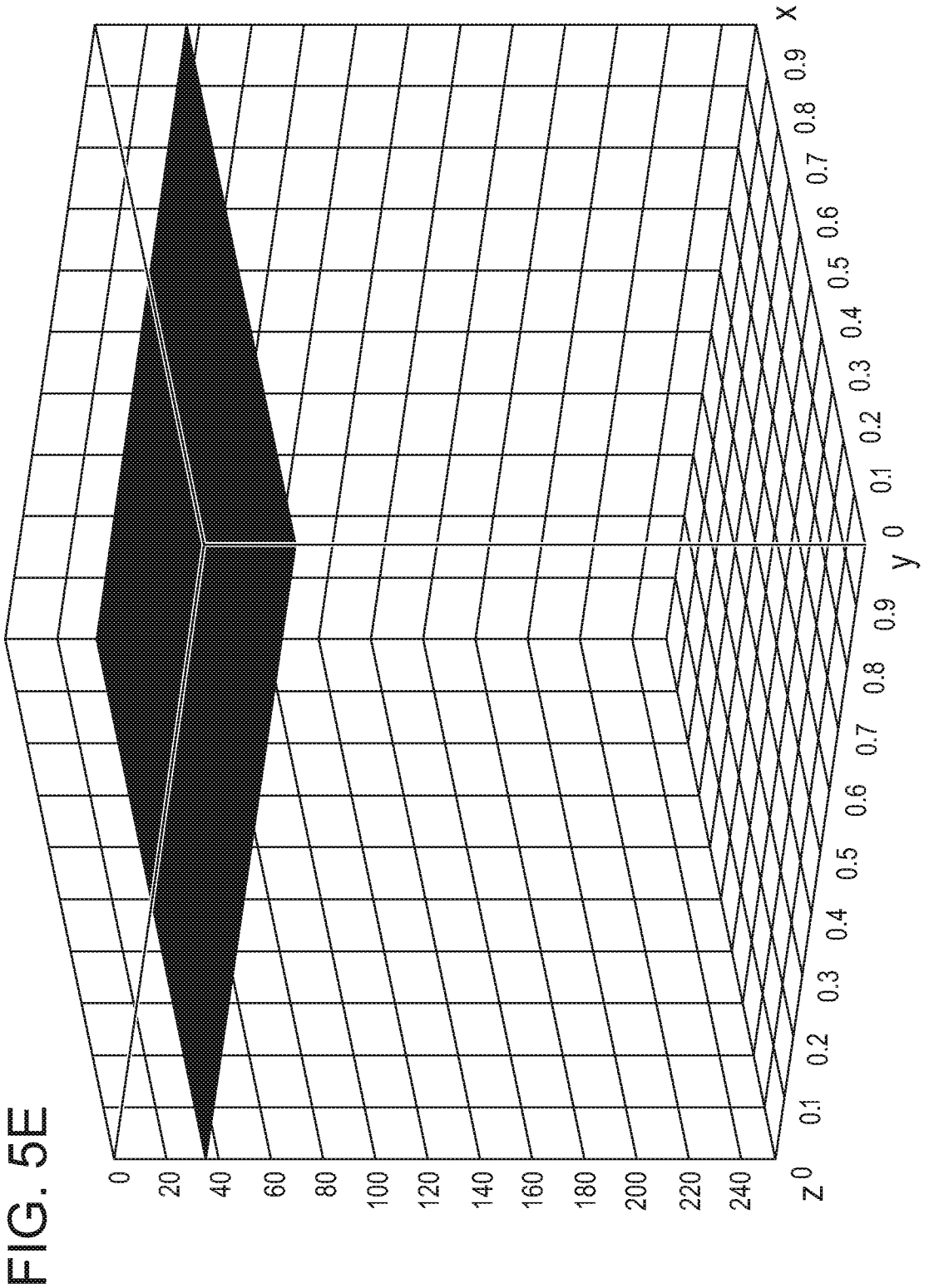

x
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
y
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
z
0 20 40 60 80 100 120 140 160 180 200 220 240

FIG. 5E

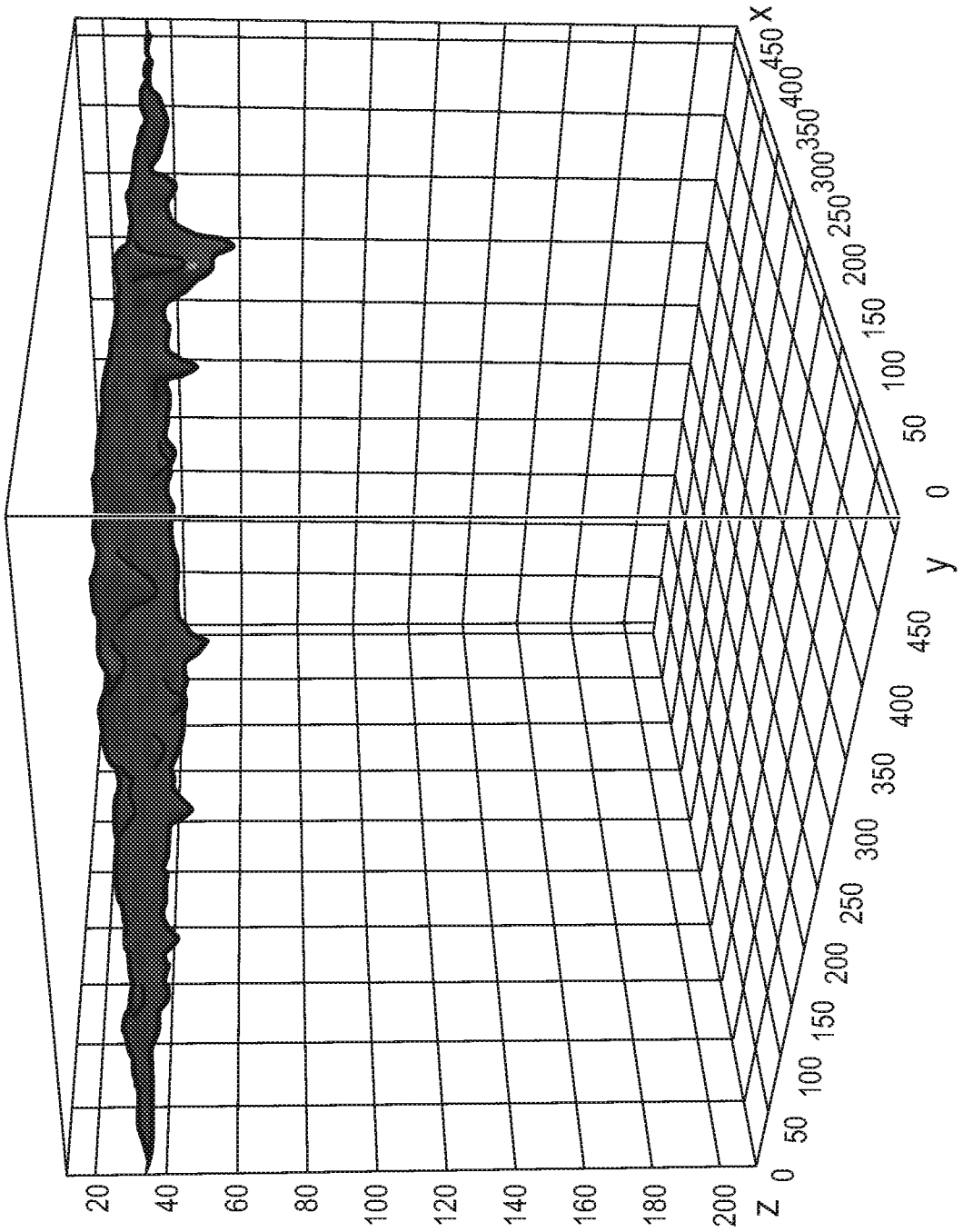

X
0
50
100
150
200
250
300
350
400
450
y
0
50
100
150
200
250
300
350
400
450
Z
20
40
60
80
100
120
140
160
180
200

FIG. 5F

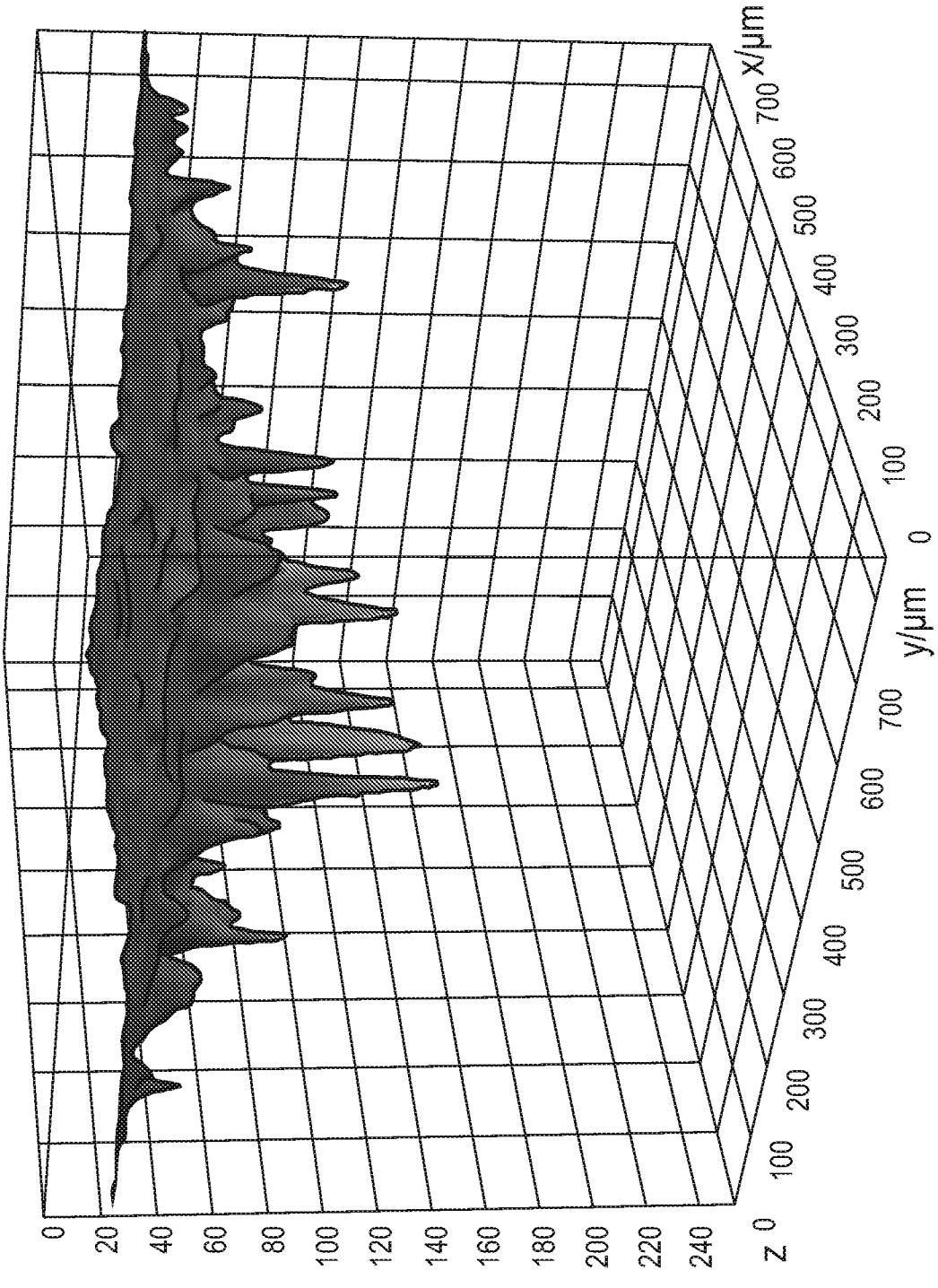

z
0
20
40
60
80
100
120
140
160
180
200
220
240
0
100
200
300
400
500
600
700
y/µm
0
100
200
300
400
500
600
700
x/µm

FIG. 5G

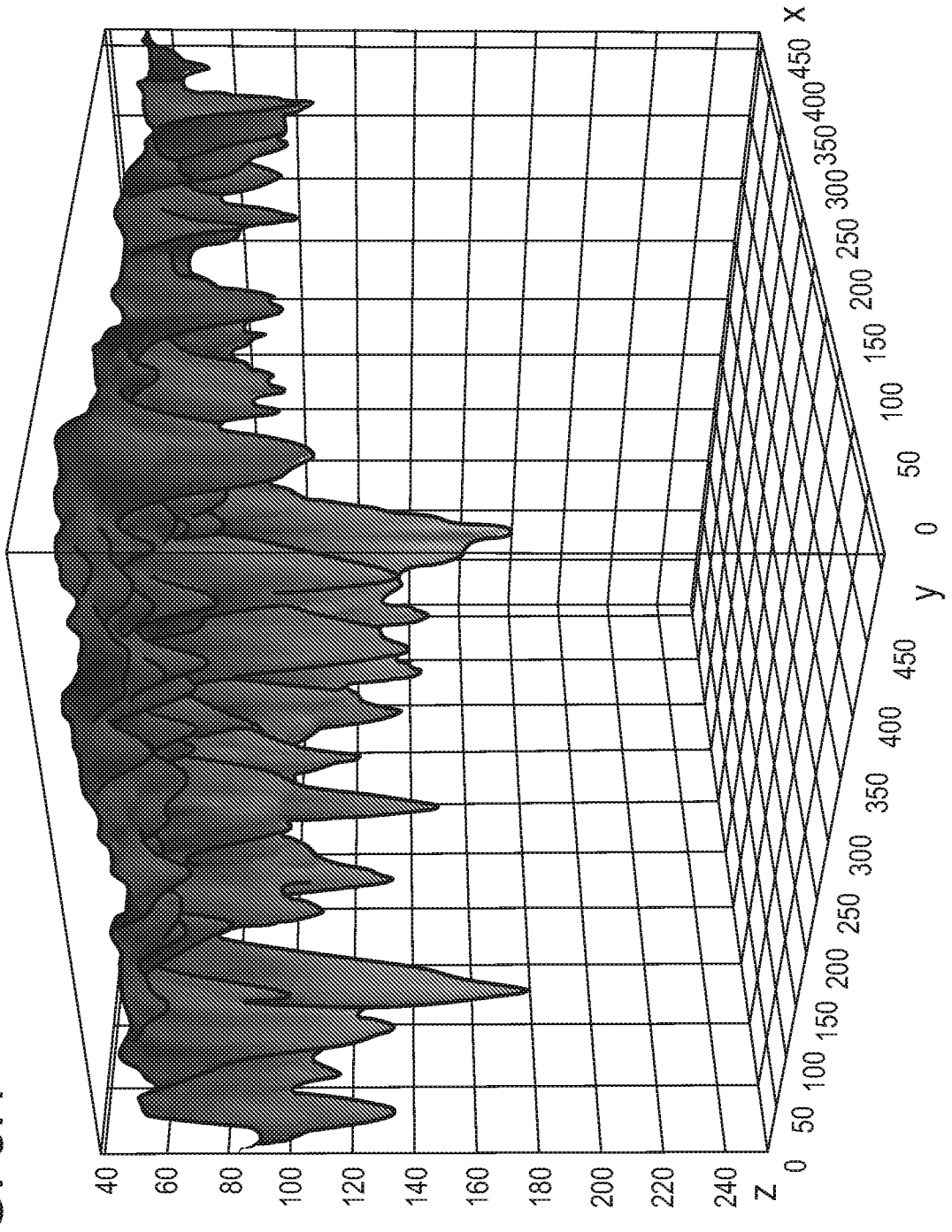

Z
40
60
80
100
120
140
160
180
200
220
240
y
0
50
100
150
200
250
300
350
400
450
X
0
50
100
150
200
250
300
350
400
450

FIG. 5H

FIG. 5O

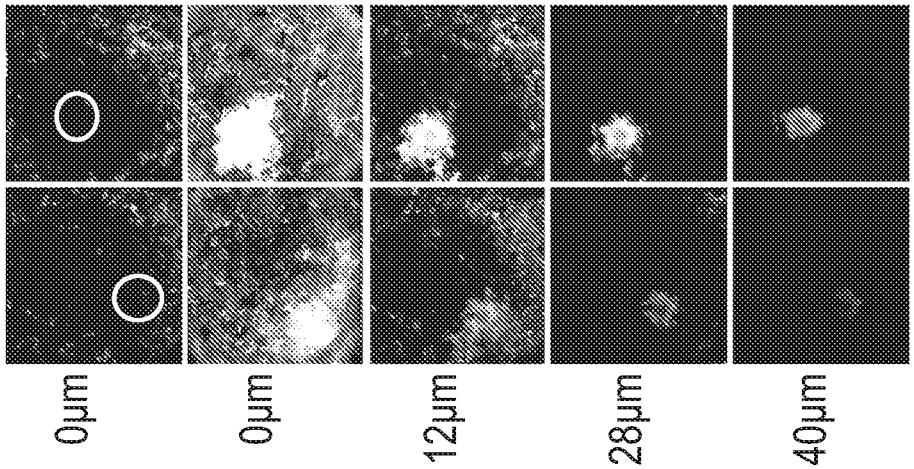


FIG. 5K GFP

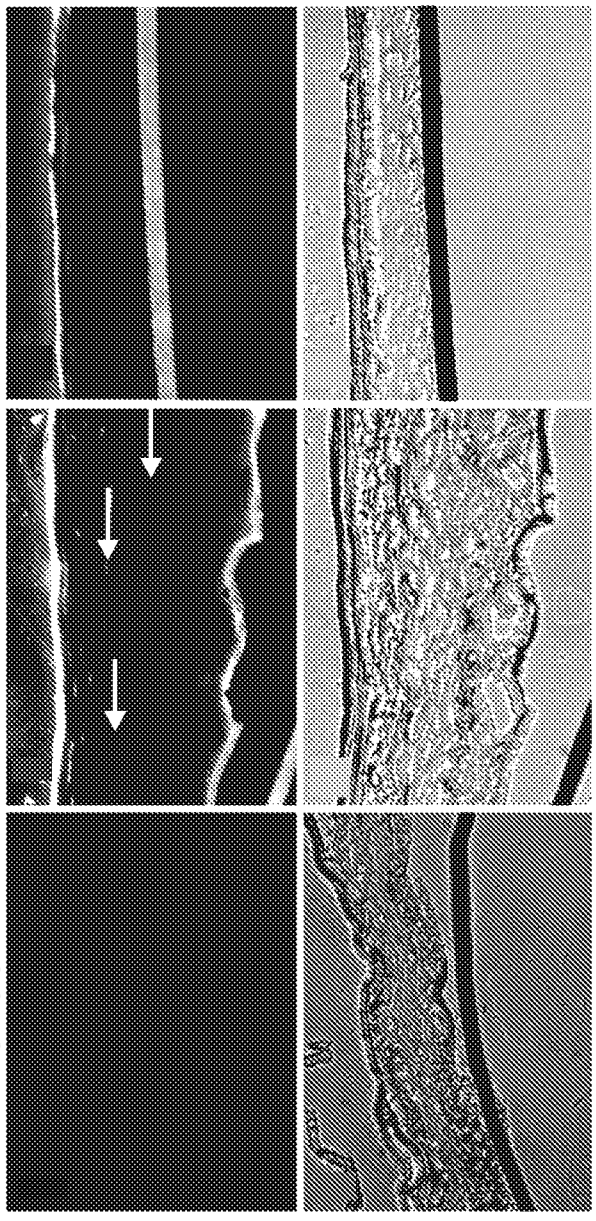
FIG. 5N

FIG. 5J GFP+RMR

FIG. 5M

FIG. 5I Vehicle

FIG. 5L



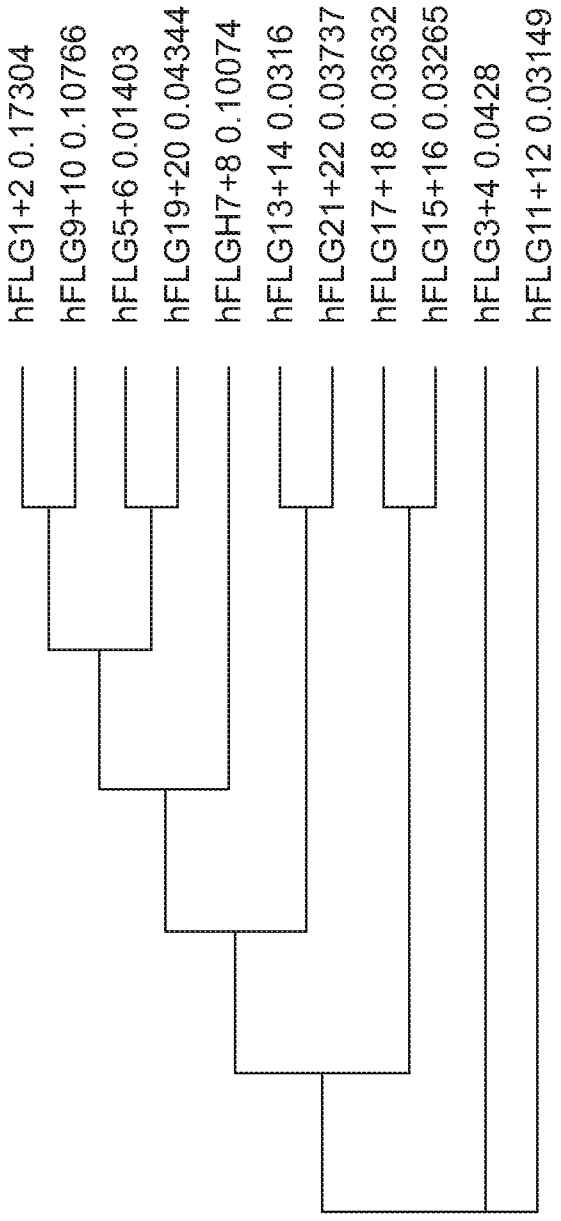
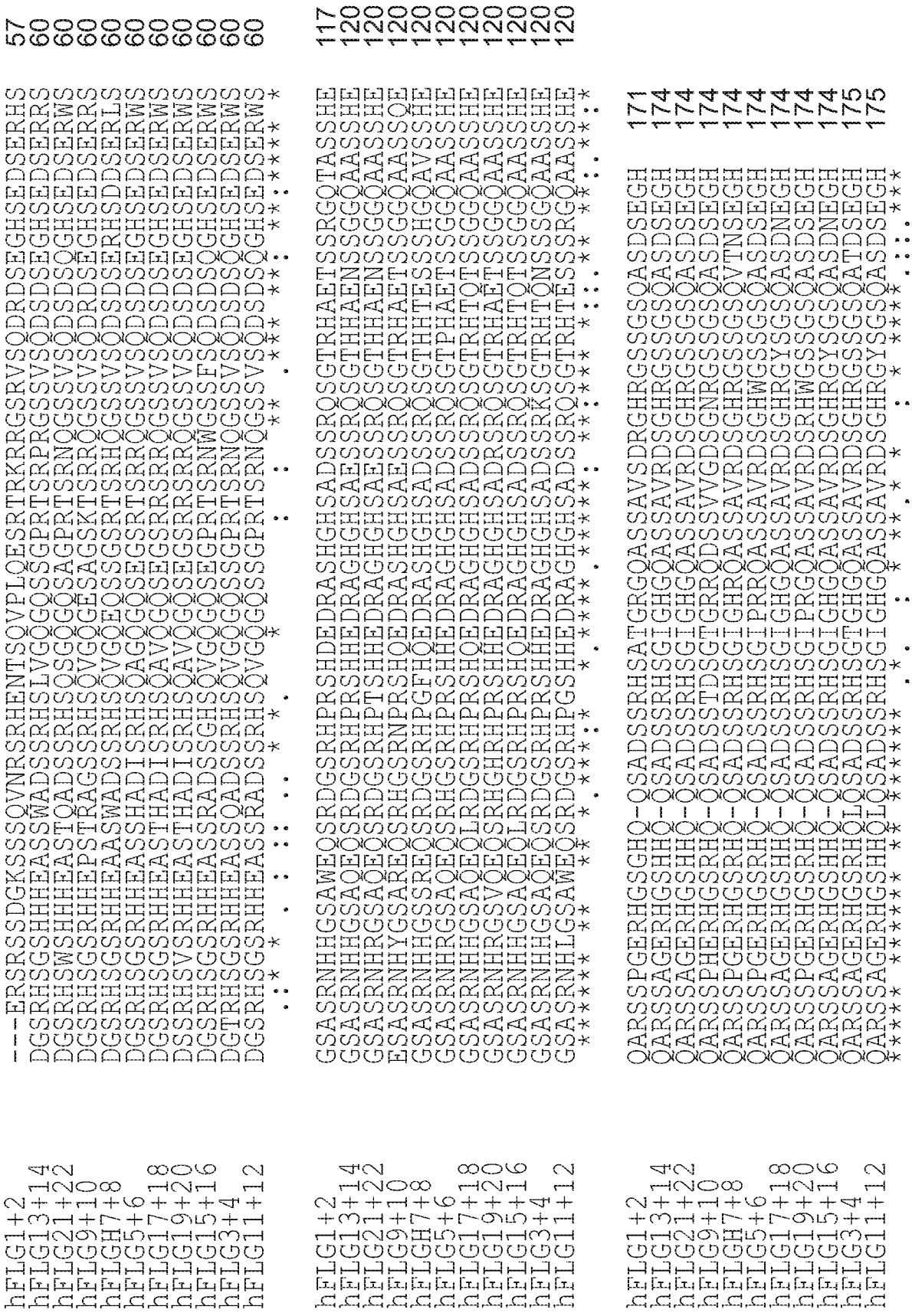

hFLG1+2 0.17304
hFLG9+10 0.10766
hFLG5+6 0.01403
hFLG19+20 0.04344
hFLGH7+8 0.10074
hFLG13+14 0.0316
hFLG21+22 0.03737
hFLG17+18 0.03632
hFLG15+16 0.03265
hFLG3+4 0.0428
hFLG11+12 0.03149

FIG. 6A

FIG. 6B



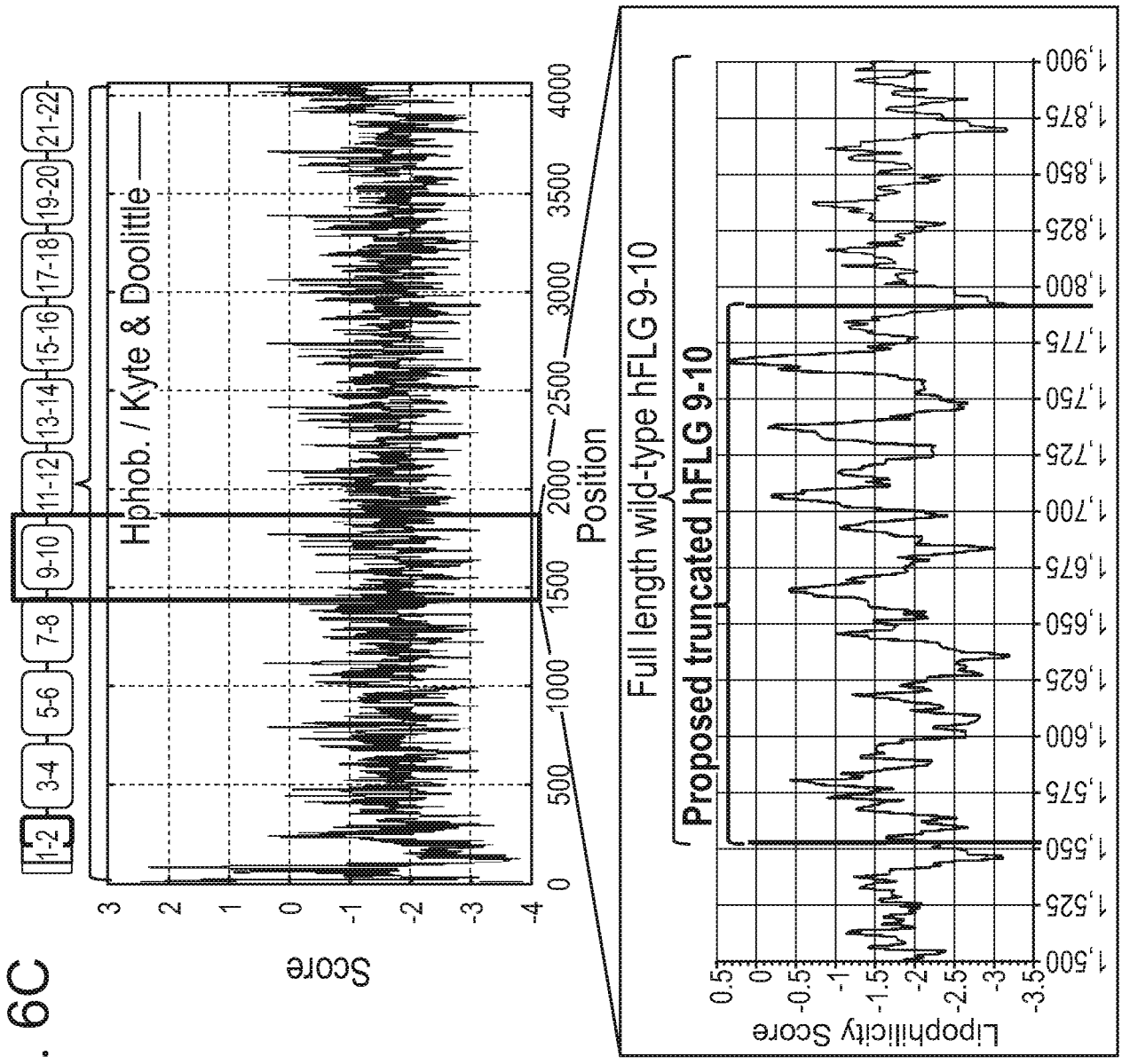

1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
19-20
21-22
Hphob. / Kyte & Doolittle
Score
Position
Full length wild-type hFLG 9-10
Proposed truncated hFLG 9-10
Lipophilicity Score

FIG. 6C

FIG. 6D

FIG. 6E

1430 1440

*M***Q** **SGE**S**SGRS**R**S**

1450 1460 1470 1480 1490 1500

FLYQVSS**HEQ** **SES**THGQTAP STGGRQGSRH EQARNSSRHS ASQDGQDTIR GHPGSSRGGR

1510 1520 1530 1540 1550 1560

QGSYHEQSVD RSGHSGYHHS HTTPQGRSDA SHGQSGPRSA SRQTRNEEQS GDGSRHSGSR

1570 1580 1590 1600 1610 1620

HHEPSTRAGS SRHSQVGQGE SAGSKTSRRQ GSSVSQDRDS EGHSEDSERR SESASRNHYG

1630 1640 1650 1660 1670 1680

SAREQSRHGS RNPRSHQEDR ASHGHSAESS RQSGTRHAET SSGGQAASSQ EGARSSPGER

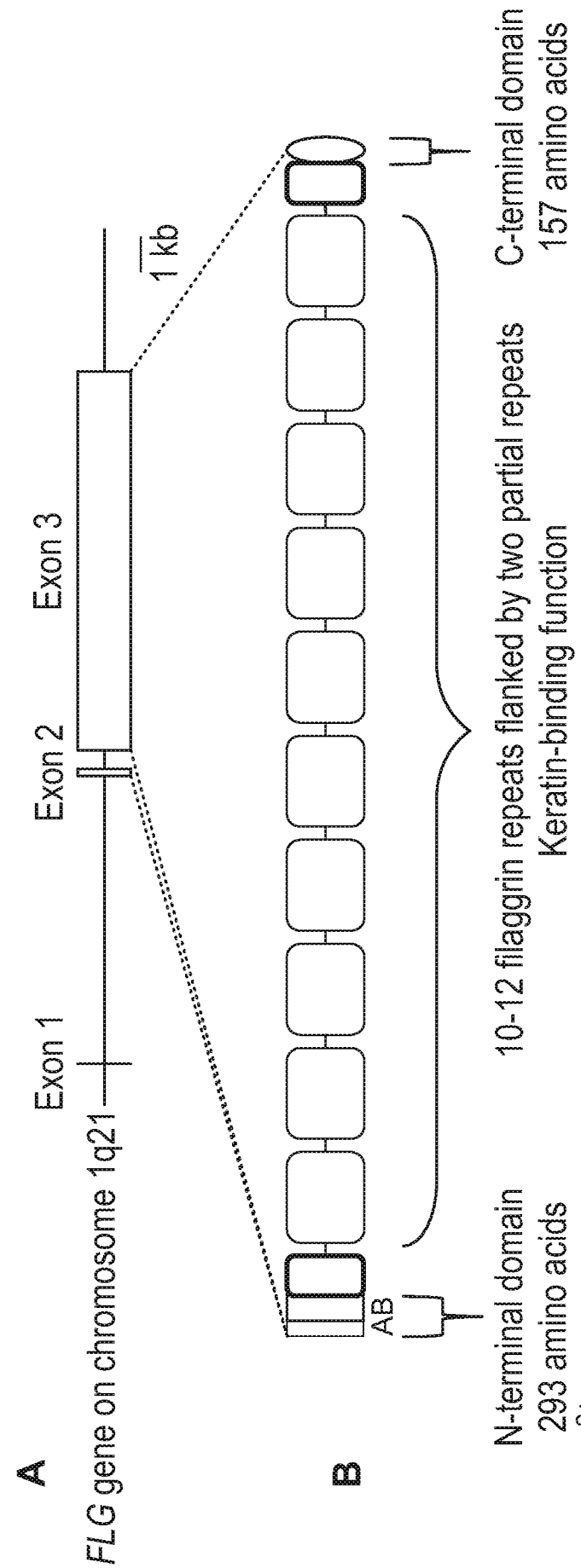
1690 1700 1710 1720 1730 1740

HGSRHQQSAD SSTDSGTGRR QDSSVVGDSG NRGSSGSQAS DSEGHSEESD TQSVSAHGQA

1750 1760 1770

GPHQQSHQES TRG**QSGE**R**SG** **RS**G**SFLYQVS** T**HEQSES***RMR* *RMRRMRR*

FIG. 6F


A
FLG gene on chromosome 1q21
Exon 1
Exon 2
Exon 3
1 kb
B
AB
N-terminal domain
293 amino acids
Ca2+-binding function
10-12 filaggrin repeats flanked by two partial repeats
Keratin-binding function
C-terminal domain
157 amino acids

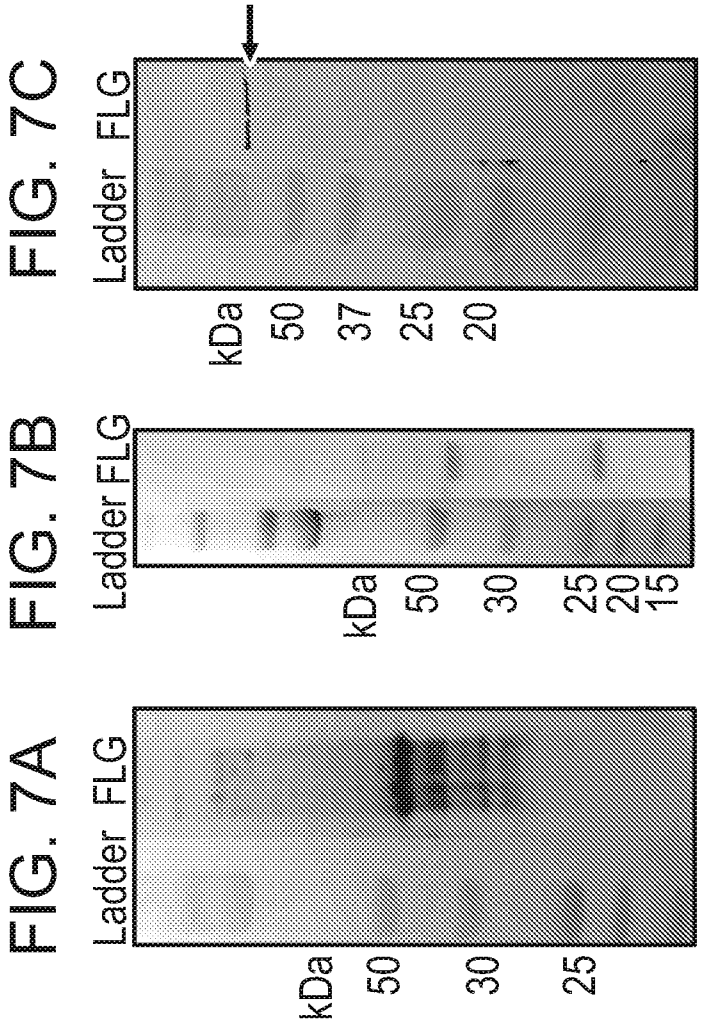


FIG. 7A

FIG. 7B

FIG. 7C

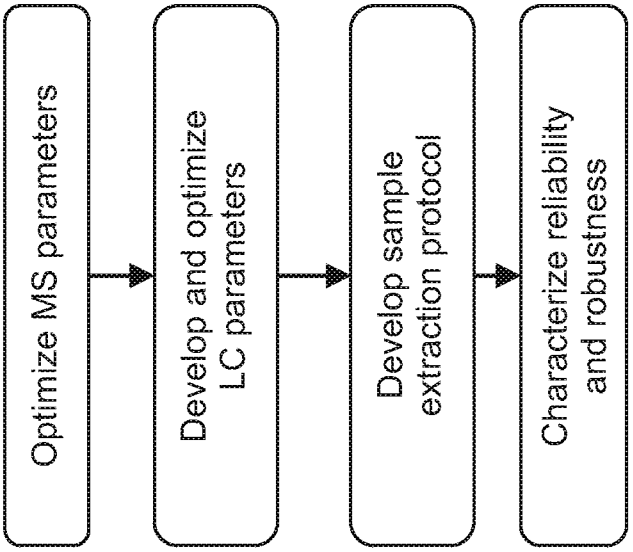


FIG. 7D

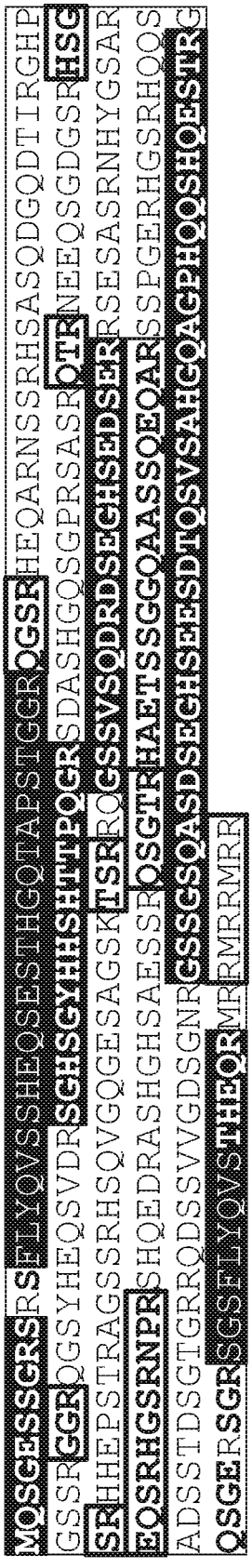


FIG. 7E

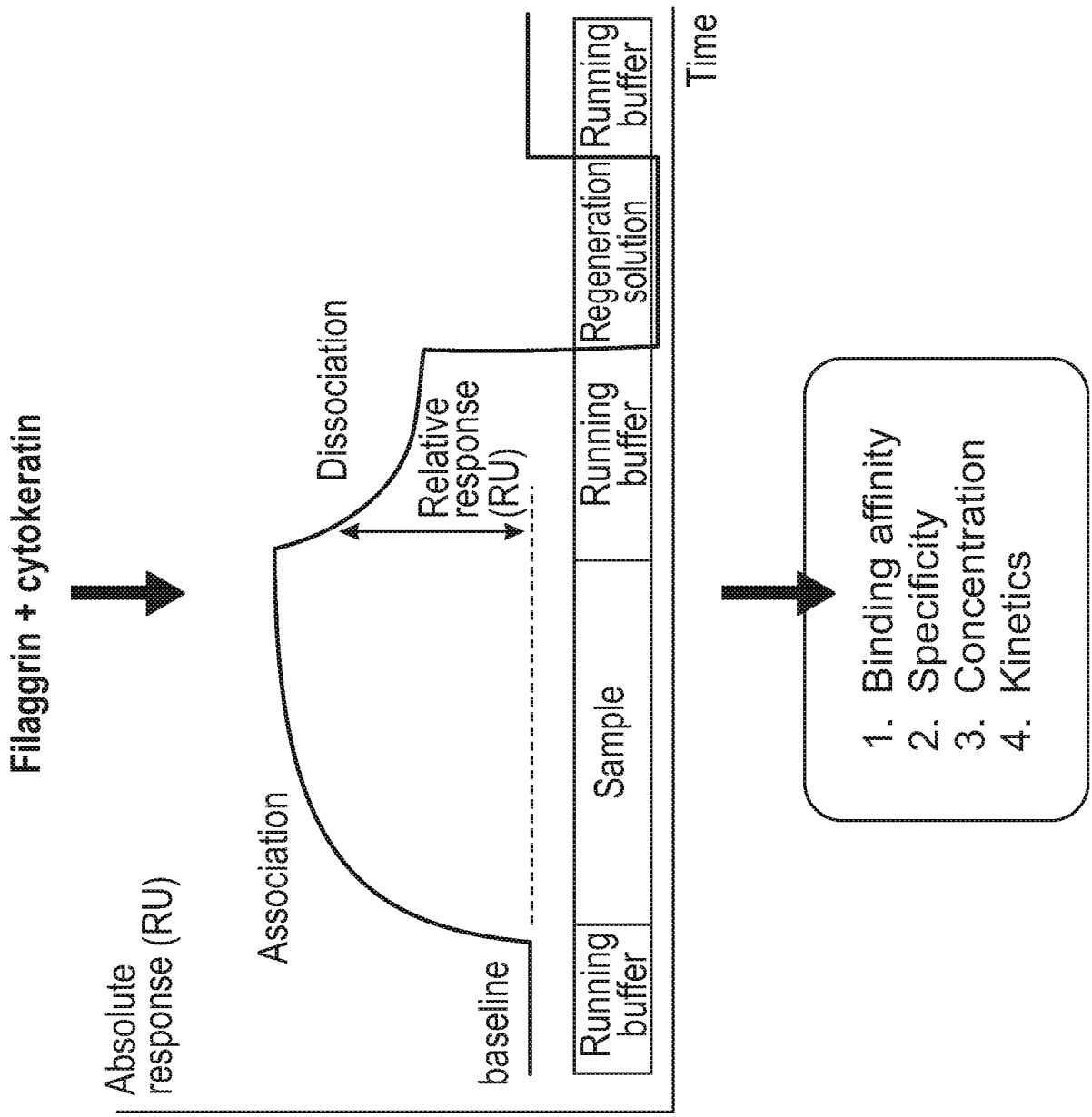

Filaggrin + cytokeratin
Absolute response (RU)
Association
Dissociation
Relative response (RU)
baseline
Running buffer
Sample
Running buffer
Regeneration solution
Running buffer
Time
1. Binding affinity
2. Specificity
3. Concentration
4. Kinetics

FIG. 8

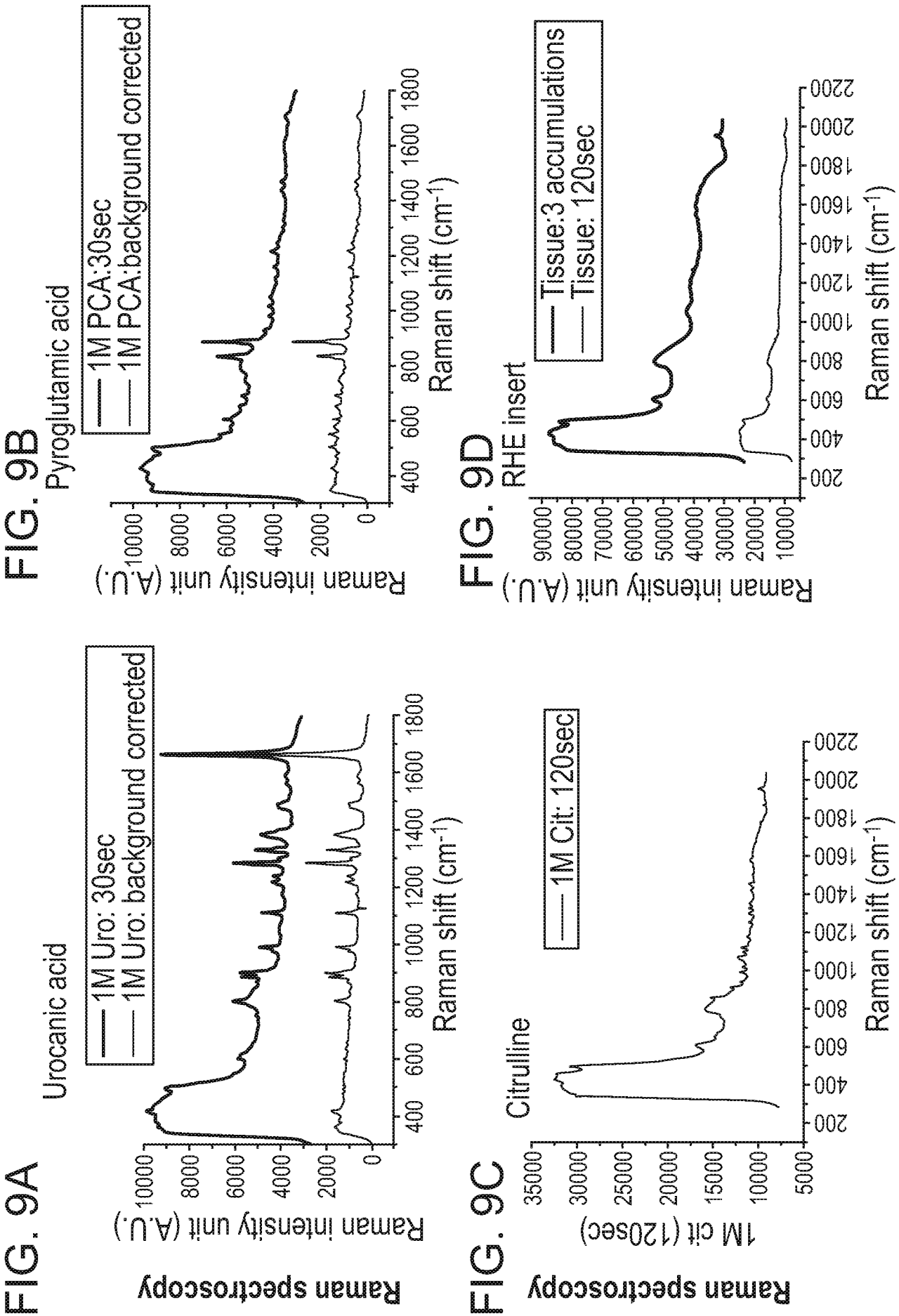


FIG. 9A
Urocanic acid
1M Uro: 30sec
1M Uro: background corrected
Raman intensity unit (A.U.)
Raman shift (cm-1)
FIG. 9B
Pyroglutamic acid
1M PCA:30sec
1M PCA:background corrected
Raman intensity unit (A.U.)
Raman shift (cm-1)
FIG. 9C
Citrulline
1M Cit: 120sec
Raman spectroscopy
1M cit (120sec)
Raman shift (cm-1)
FIG. 9D
RHE insert
Tissue:3 accumulations
Tissue: 120sec
Raman intensity unit (A.U.)
Raman shift (cm-1)

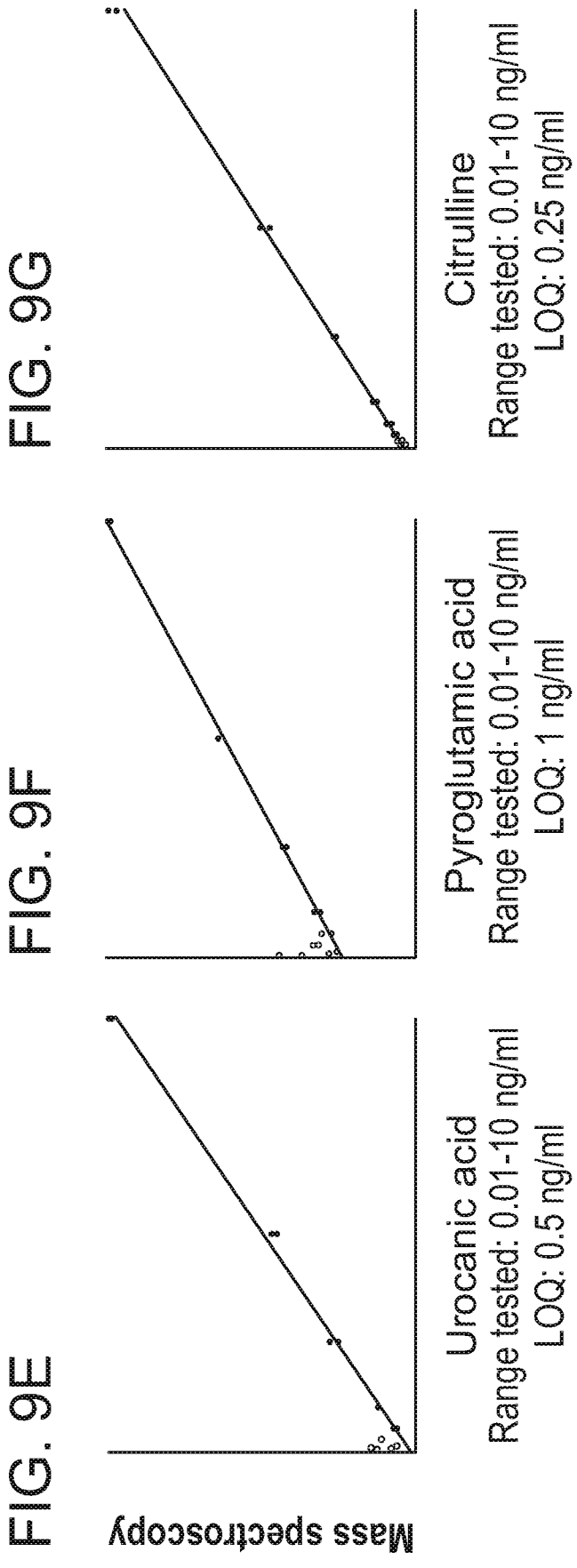
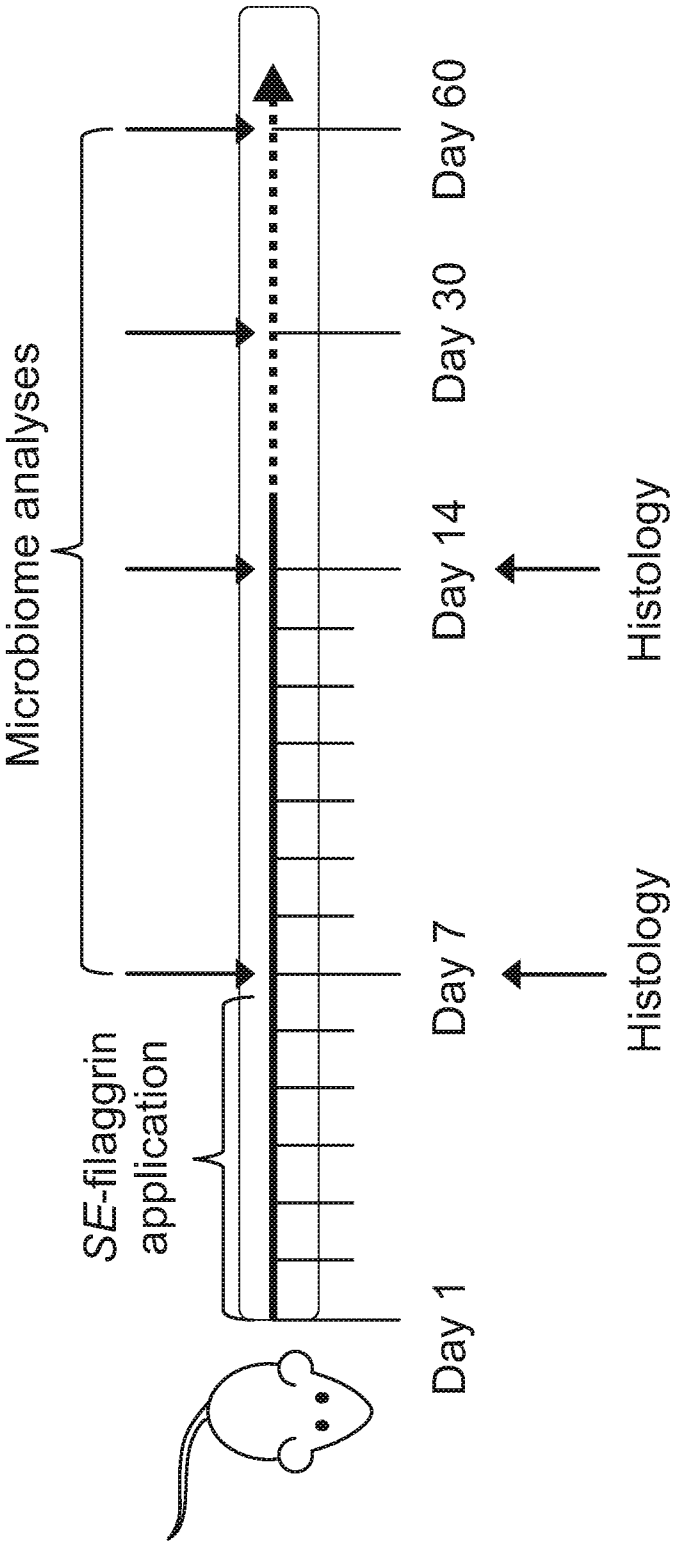


FIG. 9E

FIG. 9F

FIG. 9G

FIG. 10


Microbiome analyses
SE-filaggrin application
Day 1
Day 7
Day 14
Day 30
Day 60
Histology
Histology

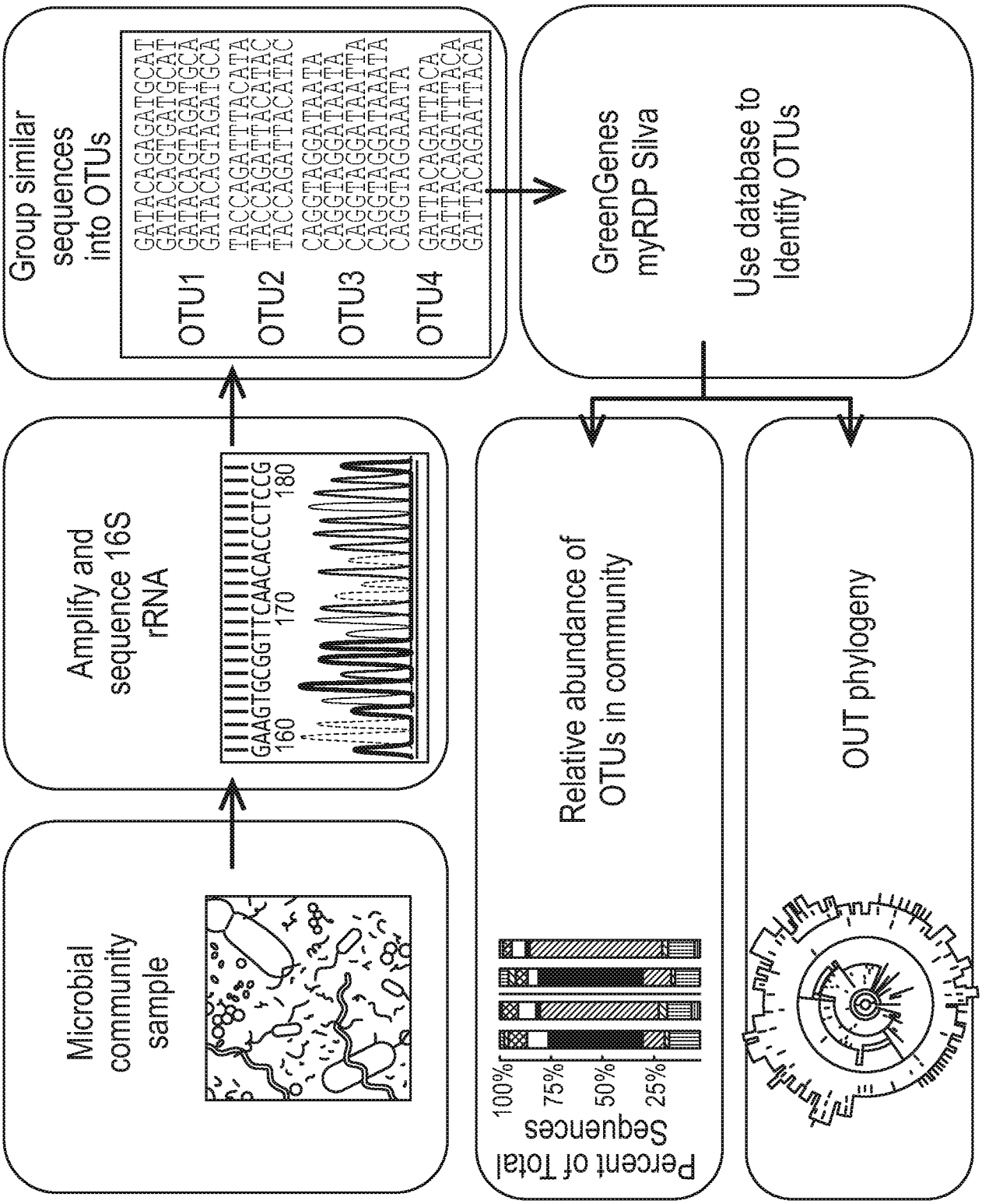
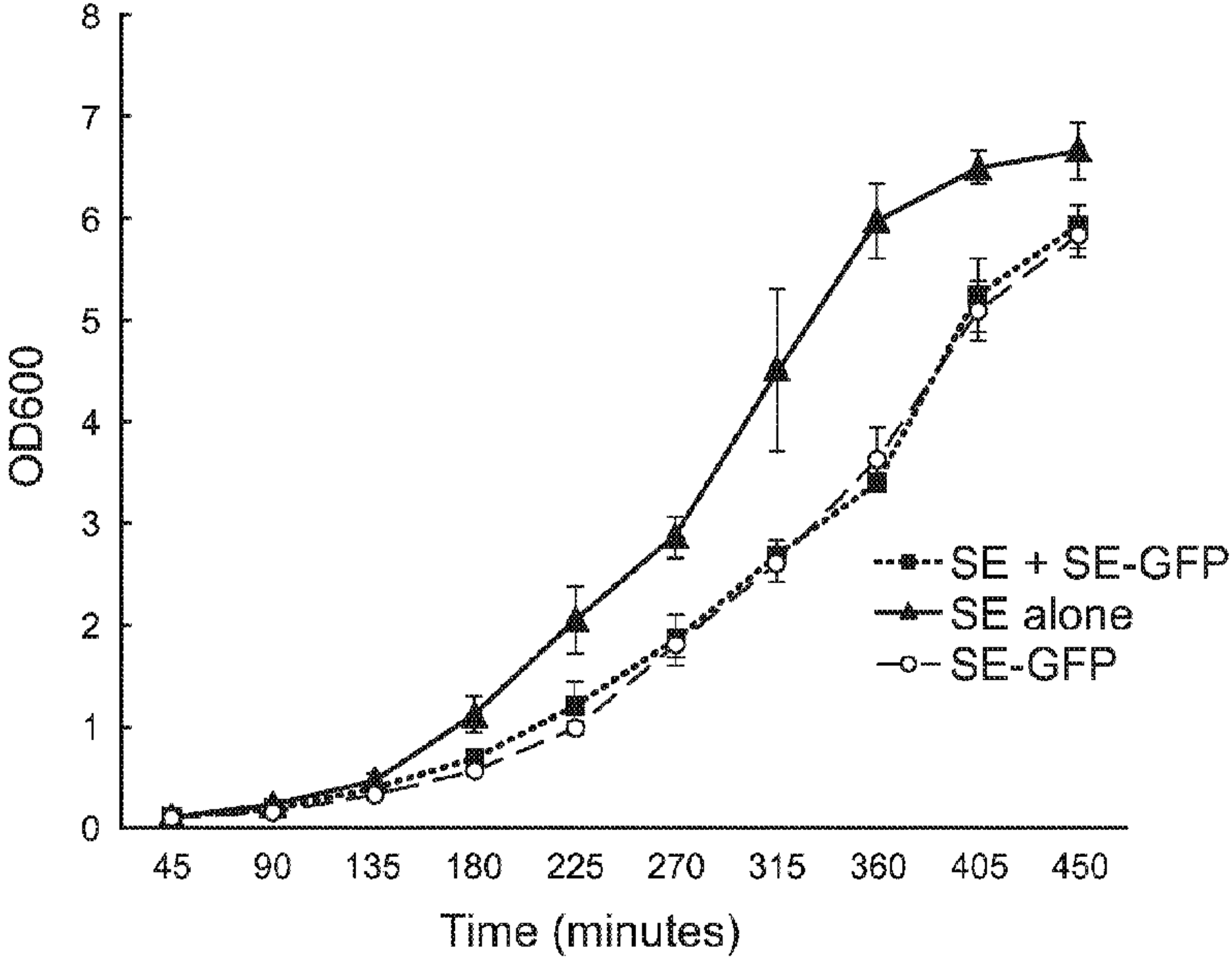

Microbial community sample
Amplify and sequence 16S rRNA
GAAGTGCGGTTCAACACCCTCCG
160 170 180
Group similar sequences into OTUs
OTU1
GATACAGAGATGCAT
GATACAGTGATGCAT
GATACAGTAGATGCA
OTU2
TACCAGATTTACATA
TACCAGATTACATAC
TACCAGATTACATAC
OTU3
CAGGTAGGATAATA
CAGGTAGGATAATTA
CAGGTAGGATAAATA
CAGGTAGGAAATA
OTU4
GATTACAGATTACA
GATTACAGATTTACA
GATTACAGAATTACA
GreenGenes
myRDP Silva
Use database to Identify OTUs
Relative abundance of OTUs in community
Percent of Total Sequences
100%
75%
50%
25%
OUT phylogeny

FIG. 11

FIG. 1


8
7
6
5
4
3
2
1
0
OD600
45
90
135
180
225
270
315
360
405
450
Time (minutes)
SE + SE-GFP
SE alone
SE-GFP