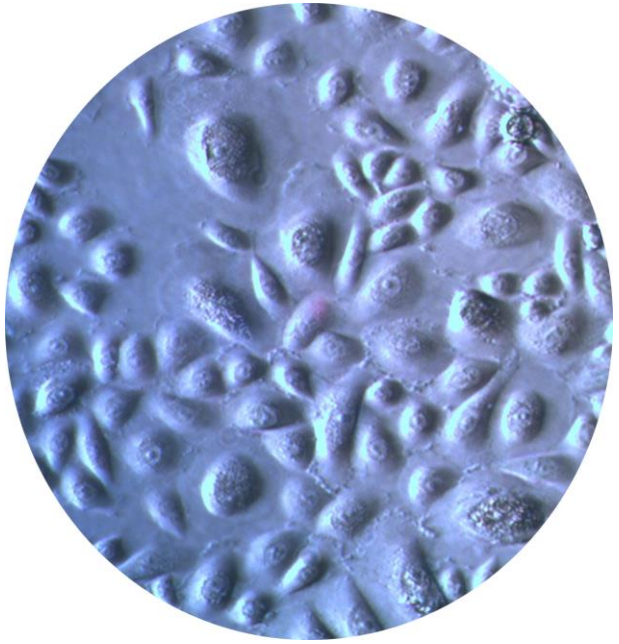
AVANTBIO™

Chemically-defined, Animal Origin-free Cell Culture For Regenerative Medicine and Other Applications



Historically, Many Cell- and Tissue-based Therapy Protocols Utilize Cell Culture Media that Includes the Use of Components that are Originated from Human or Non-human Animal (e.g. bovine, porcine, rodent) Sources. These Animal-derived Components Include:

- *Blood-derived Serum and Plasma*
- *Serum-derived Albumin, Transferrin and Fetuin Protein*
- *Replication Incompetent Feeder Layer Cells (both mortal and immortal cell lines)*
- *Pituitary Gland Tissue Extract*
- *Placental Amniotic Membranes*
- *Extracellular Matrix Proteins*
- *Blood-derived Platelet Lysate*

Motivation for Cell- and Engineered Tissue-based Therapy Companies to Adopt Chemically-defined Animal Origin-free (cdAOF) Cell Culture Systems:

- *Lessens the risk of animal component originated adverse events, that include the transfer of animal-originated pathogens (e.g., mad cow/BSE, viruses) to humans as well as inflammatory (allergic) reactions to animal-derived cell culture media components*

- *Reduces the potential for cell and tissue therapy product variability by using quality-tested, controllable, chemically defined cell culture components (i.e. precise control of cell and tissue culture manufacturing conditions)*

- *Pressure from regulatory authorities (FDA, EMA) to pursue cdAOF Cell Culture Options*

- *Can reduce the cost of manufacturing therapeutic cells and tissue, especially in light of recent qualified fetal bovine serum (FBS) shortages as well as the increasing cost for qualified FBS and other animal components*

- *Pressure from animal welfare groups and the general public to employ cdAOF cell culture technology, as it is also "Animal Cruelty-free"*

- *Provides a competitive marketing advantage that highlights complete "animal cruelty-free" (e.g. fetal bovine serum-free) cell and tissue culture environment when carrying out the in vitro testing and marketing of OTC cosmeceuticals and other consumer personal care products*

To Avoid the Use of Animal Derived Components for The Culture of Human Skin-derived Epidermal Keratinocytes, Avantbio Developed an Improved, Chemically-defined Animal Origin-free (cdAOF) Cell Culture Supplement System That Does Not Include any Human or Other Animal-derived Products. The Following is a Collection of Representative Research and Development Results Derived from the Testing of This New Clinically-relevant cdAOF Cell Culture System.

HKSdaFREE KIT

A 2-component supplement kit

(HKSdaFREE & HKGE)

AVANTBIO™

Chemically-defined, Animal Origin-free (cdAOF) Cell and Tissue Culture Systems

Slides 6-11 Primary Isolation and Serial Propagation of Neonatal and Adult Human Epidermal Keratinocytes (HEKn and HEKa)

Slides 12-13 Improved HEKn Proliiferative Performance Over Supplement S7

Slide 14 Beta Site Study Independently Demonstrates Improved HEKn Proliferative Performance Over Supplement S7

Slides 15-16 Reconstructed Human Epidermis (RHE)

Slides 17-20 HEKs as a Novel Bioink for an Implantable 3D Bioprinted full-thickness Skin Equivalent

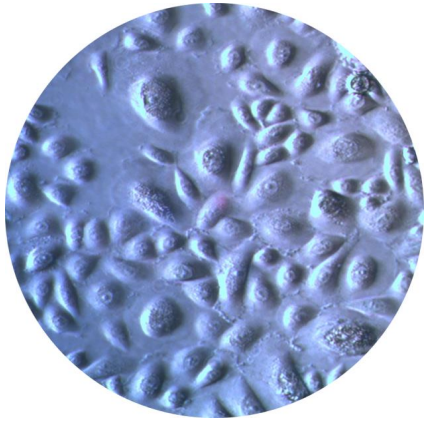
Slides 21-24 Primary Isolation and Serial Propagation of Adult Corneal Epithelial Cells (HCEC)

Slides 25-26 Reconstructed Human Corneal Epithelium (RHCE)

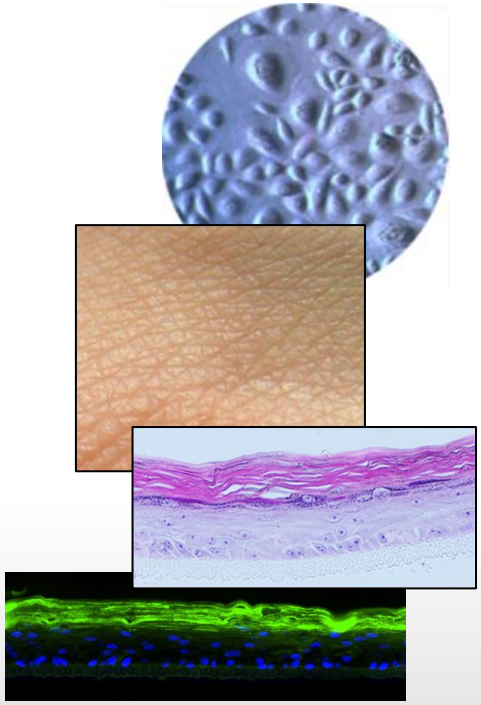
Slides 27-29 Propagation of HEKn and HCEC on a Novel Bio-compatable Substratum: Silk-derived Fibroin Discs (Mats)

Slides 30-31 Propagation of BPE-starved Human Gingival Epithelial Cells

Slides 32-34 Propagation of the MDCK and VERO Immortalized Cell Lines



Human Epidermal Keratinocytes (HEKn and HEKa)



Potential Applications in Basic and Preclinical Laboratory Research

- ***2D Epithelial Cell Cultures***
  - ***Human Epidermal Keratinocytes***
- ***3D Bioprinted, Automated or Manually Reconstructed Tissue, Using Human Keratinocytes***
  - ***3D Reconstructed Epidermis***
  - ***3D Reconstructed Epidermis on a Chip***
  - ***3D Reconstructed Epidermis for In Vitro Testing (e.g., Irritancy, Corrosion, Genotoxicity, Permeation, Other)***

Potential Clinical Applications in Regenerative and Esthetic Medicine Using Keratinocyte Cell Cultures

- ***2D Cell-based Cutaneous Therapies***
- ***3D Reconstructed Tissue-based Cutaneous Therapies***

Improved, Defined, Animal Origin-free (dAOF) Primary Isolation and Serial Propagation of Human Keratinocytes, Human Corneal Epithelial Cells and Other Clinically-relevant Cell Types.

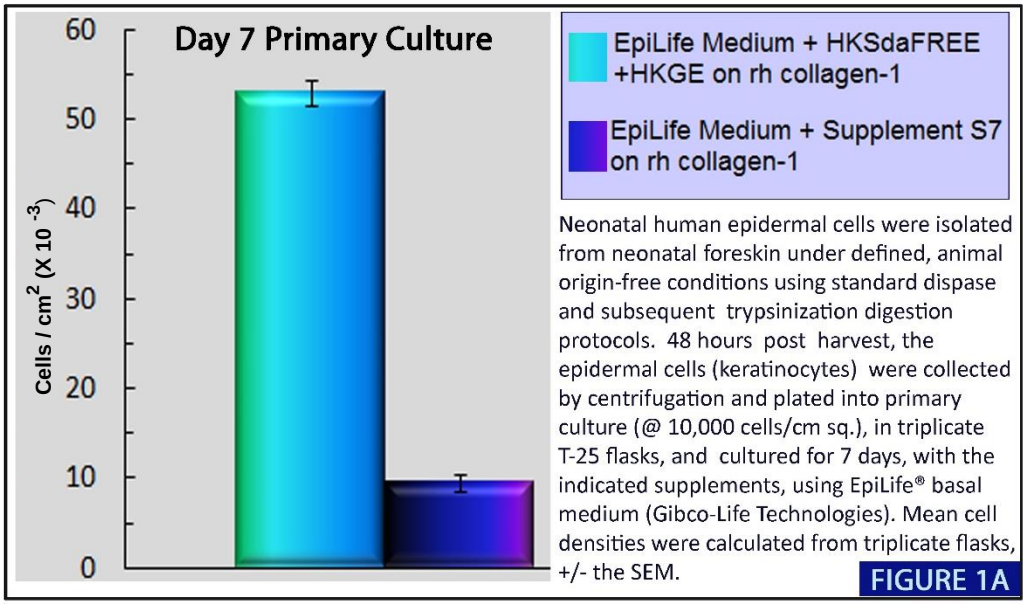
Paul W Cook, Rolf W Winter and Jeffrey R Brown. AvantBio Corporation, Vancouver WA USA

J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012
Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC

dAOF cell culture can reduce or eliminate xenopathogen transmission from animal cell culture components (e.g. sera, BPE, serum derived BSA, murine feeder cells) to humans undergoing either cell- and engineered tissue-based cutaneous and ocular therapies. Improved dAOF cell culture systems for human neonatal keratinoctyes (HEKn), adult keratinoctyes (HEKa) and corneal epithelial cells (HCEC) are of particular interest. We have recently developed a new dAOF HEK cell culture supplement system **(HKSdaFREE + HKGE or HKGE2)** that supports the primary isolation and serial propagation of HEKn, HEKa, and HCEC, in a complete dAOF cell culture environment, using commercially-available HEK basal media (e.g. EpiLife© or KBM©). For HEKn cultured in EpiLife©, over 83 and 90 cumulative post-primary population doublings (cPDLs) can be attained on untreated and recombinant human collagen-1 pretreated substrata, respectively. Compared to predecessor dAOF HEK cell culture systems, post-primary propagation is not strictly dependent on precoating the cell culture surface, and both replicative lifespan and doubling rates are increased (>1 PDL/day up to day 76). **Our new dAOF HEK culture system also supports the generation of reconstructed human epidermis under dAOF culture conditions.** Using EpiLife© basal medium, adult HCECs display similar growth characteristics (>40 cPDLs & >1 PDL/day up to day 39), and can be propagated at the post-primary level, with or without substratum pretreatment. Recent results suggest that our new dAOF supplement system can support the primary and post-primary propagation of human cornea-derived fibroblasts (keratocytes) and corneal endothelial cells. The dAOF culture of human dermal fibroblasts, mesenchymal stem cells and hair follicle dermal papilla cells are under investigation. Collectively, our new dAOF cell culture system may be adaptable to a variety of cell types, raising the possibility for routine use of dAOF cells in the repair of damaged or diseased human tissues.

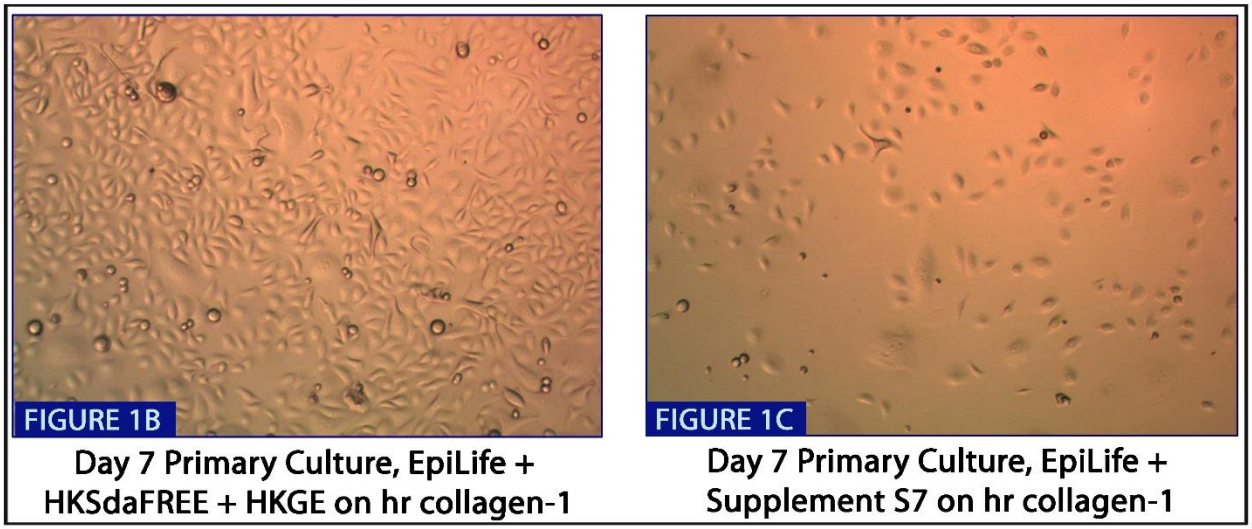
AvantBio's HKSdaFREE KIT Supplements Improve the Isolation and Propagation of Primary Neonatal Human Epidermal Keratinocytes (HEKn) Under Chemically Defined Animal Origin-free (cdAOF) Conditions.

From J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012 Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC



Neonatal human epidermal cells were isolated from neonatal foreskin under defined, animal origin-free conditions using standard dispase and subsequent trypsinization digestion protocols. 48 hours post harvest, the epidermal cells (keratinocytes) were collected by centrifugation and plated into primary culture (@ 10,000 cells/cm sq.), in triplicate T-25 flasks, and cultured for 7 days, with the indicated supplements, using EpiLife® basal medium (Gibco-Life Technologies). Mean cell densities were calculated from triplicate flasks, +/- the SEM.

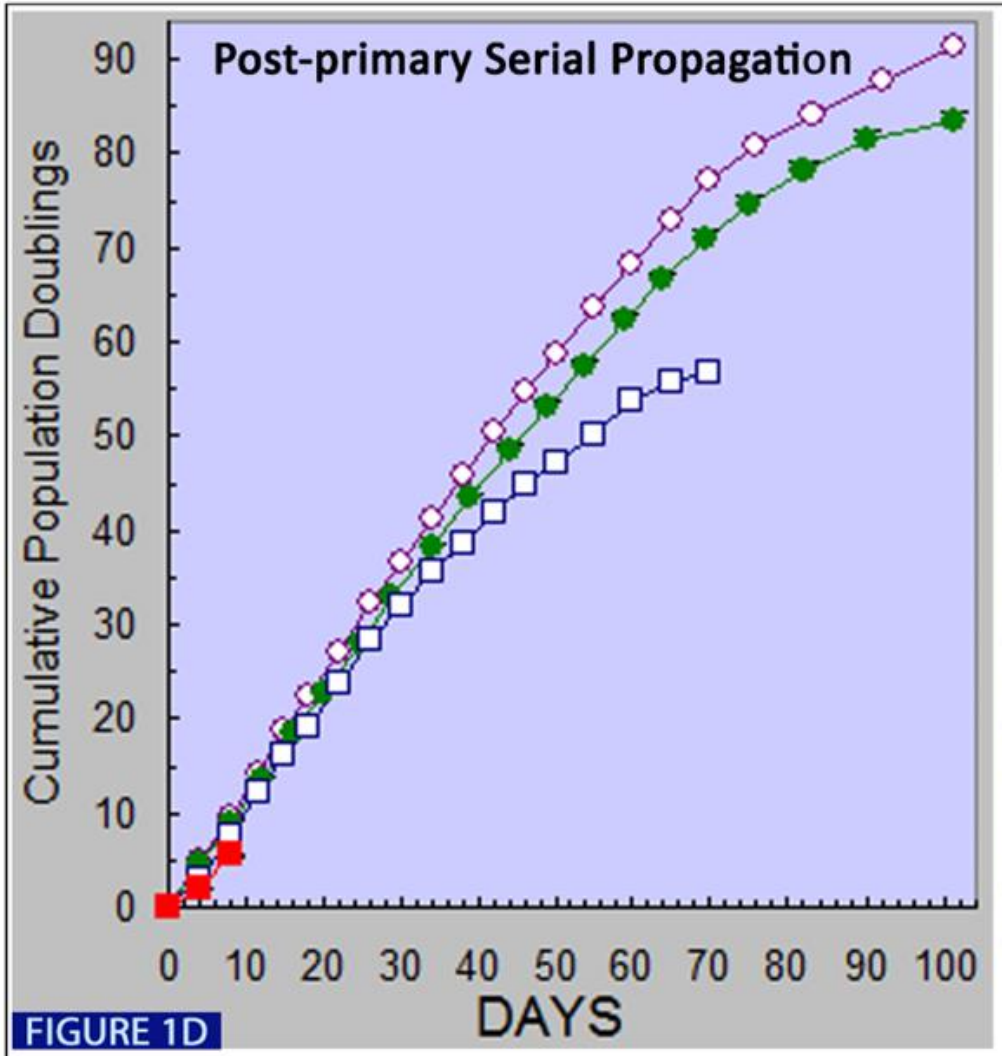
FIGURE 1A

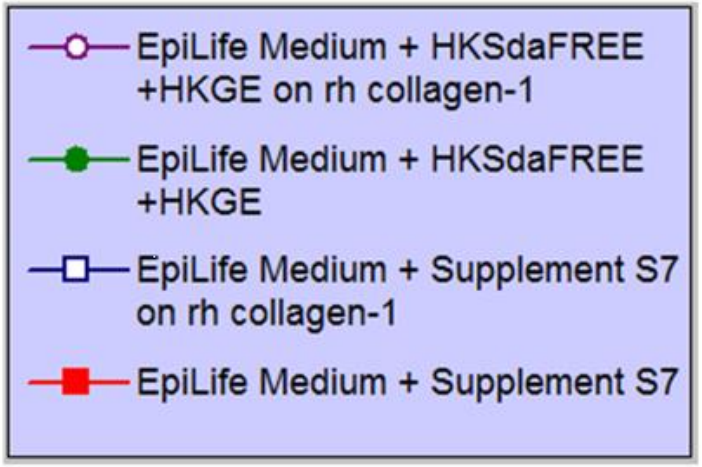


EpiLife and Supplement S7 are trademarks of Life Technologies Corporation.

AvantBio's HKSdaFREE KIT Supplements Improve the Post-primary Serial Propagation of Neonatal Human Epidermal Keratinocytes (HEKn) Under Chemically Defined Animal Origin-free (cdAOF) Conditions. Efficient Serial Propagation is Not Dependent on Pre-coating the Cell Culture Surface with Recombinant Human Collagen-1 (rh Collagen-1).

From J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012 Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC



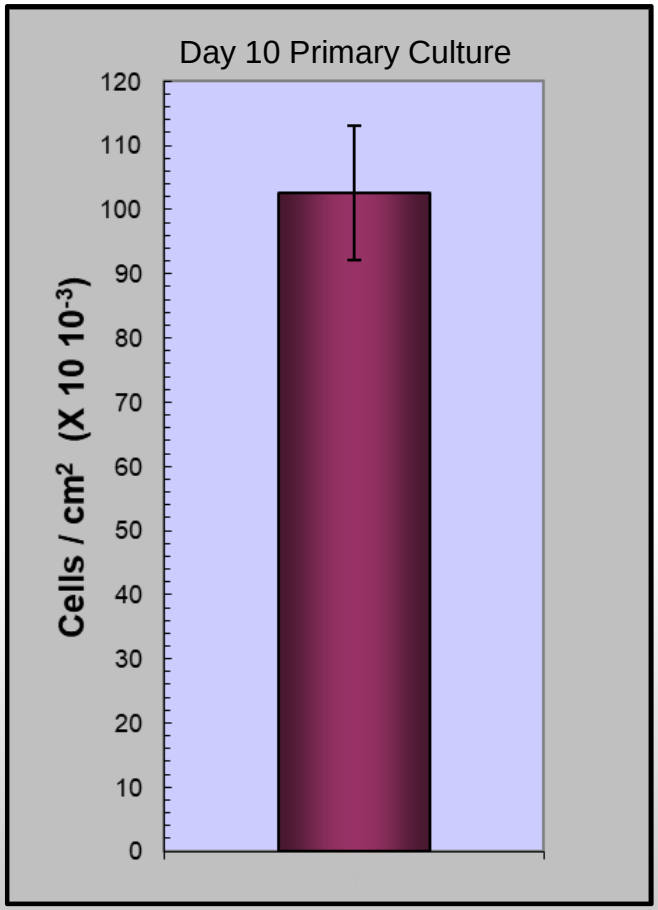


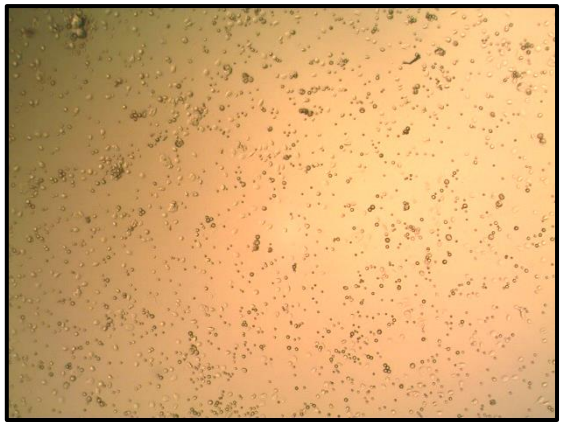
Defined, animal origin-free (dAOF) neonatal human keratinocyte primary cell cultures (as described above in figures 4 A-C) were dissociated under dAOF conditions and plated into passage 1- 21 culture (at 2,500 viable cells/cm sq.), in duplicate T-25 flasks, and serially passaged in the dAOF cell culture environment using the indicated dAOF supplements and EpiLife® basal medium (Gibco-Life Technologies). Values represent mean cumulative population doublings, +/- SEM.

EpiLife® and Supplemment S7® are registered trademark of Life Technologies

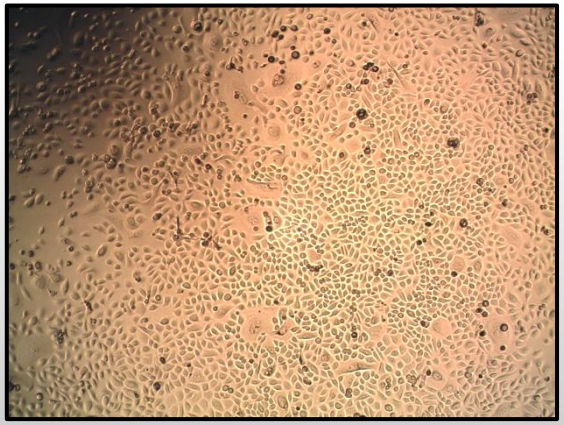
AvantBio's HKSdaFREE KIT Supplements Enable the Efficient Isolation and Propagation of Primary Adult Human Epidermal Keratinocytes (HEKa) Under Chemically Defined Animal Origin-free (cdAOF) Conditions.

From J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012 Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC

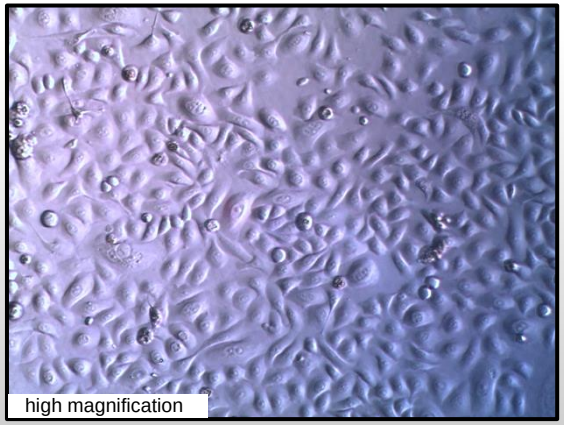




Primary Culture Day 1 Photomicrograph



Primary Culture Day 10 Photomicrograph

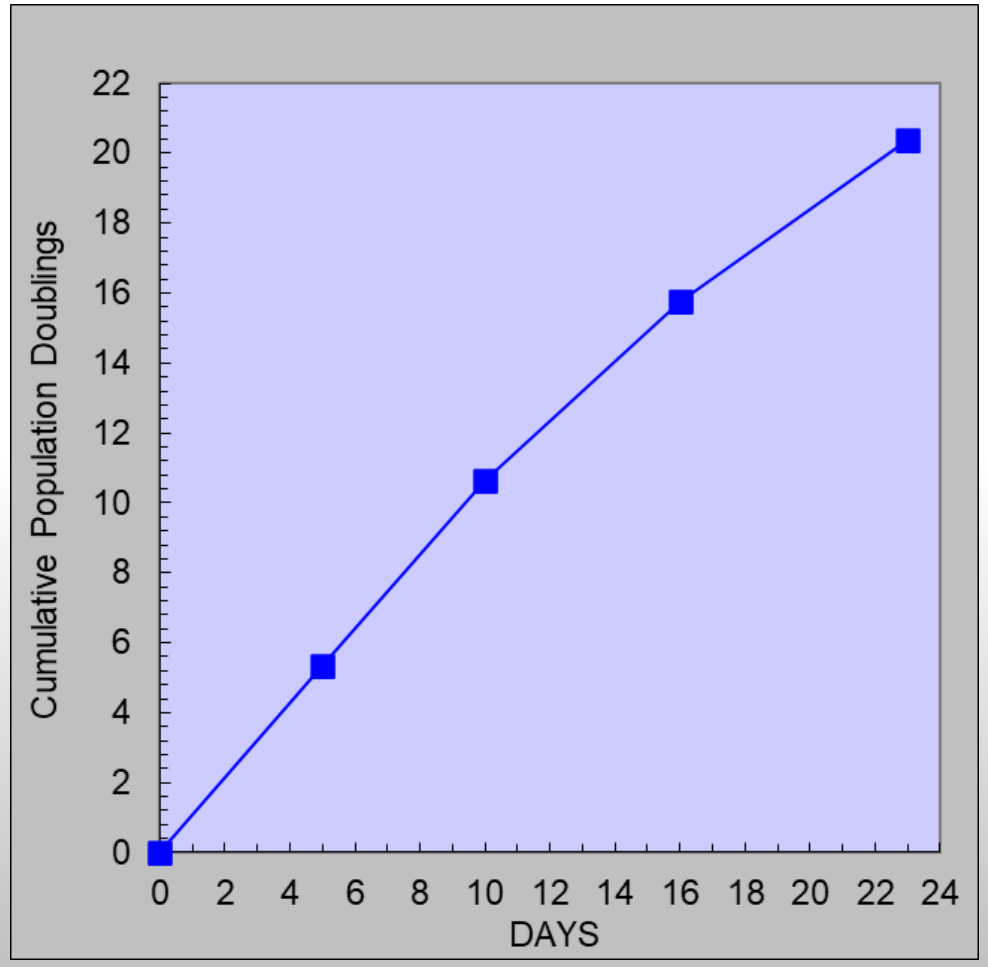


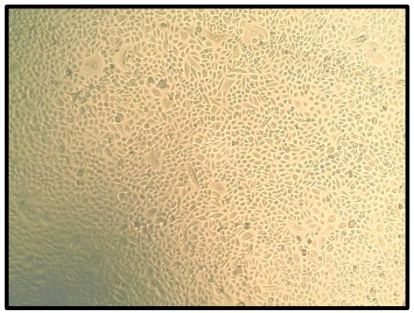
Primary Culture Day 10 Photomicrograph

Methods: Primary adult human epidermal keratinocytes cultures (HEKa) were isolated from adult skin punch biopsies (1.13 cm^2 from a 53-year-old female) and placed into chemically defined animal origin-free (cdA0F) cell culture conditions. Briefly, utilizing a protocol employing 21-hour dispase treatment at 4 deg. C., the epidermal cells were collected by removal of the dissociated epithelium, enzymatic dissociation with TrypLE Select and centrifugation. 36 hours post-tissue harvest, $1.62x10^6$ viable epidermal cells were recovered and some of the viable epidermal cells were plated into primary culture (@ 30,000 viable cells / cm^2, in quadruplicate, in rh Collagen-1 coated 2 cm^2 cell wells. The cells were cultured for 10 days using EpiLife basal medium and AvantBio's HKGSdaFREE KIT supplements. Media was exchanged every 2 days. Cell densities at day 10 were determined by TrypLE Select enzymatic harvesting and manual cell counting. Mean cell densities were calculated from quadruplicate wells, +/- the SEM. EpiLife is a trademark of Life Technologies Corporation.

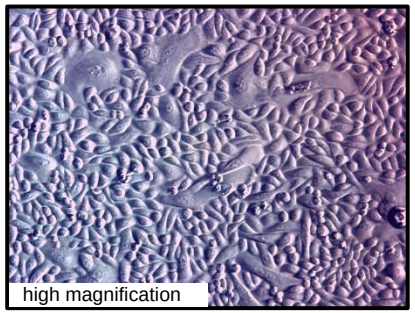
AvantBio's HKSdaFREE KIT Supplements Enable the Efficient Serial Propagation of Post-primary Adult Human Epidermal Keratinocytes (HEKa) Under Chemically Defined Animal Origin-free (cdAOF) Conditions.

From J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012 Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC

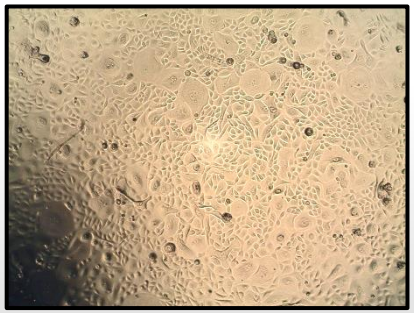




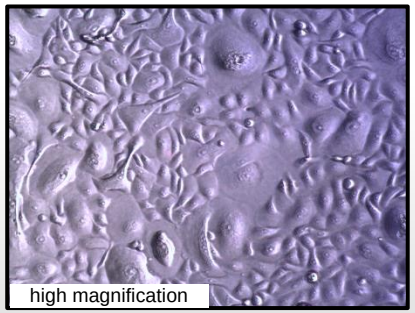
Post-primary Passage 1,
Day 5 Photomicrograph



Post-primary Passage 1,
Day 5 Photomicrograph



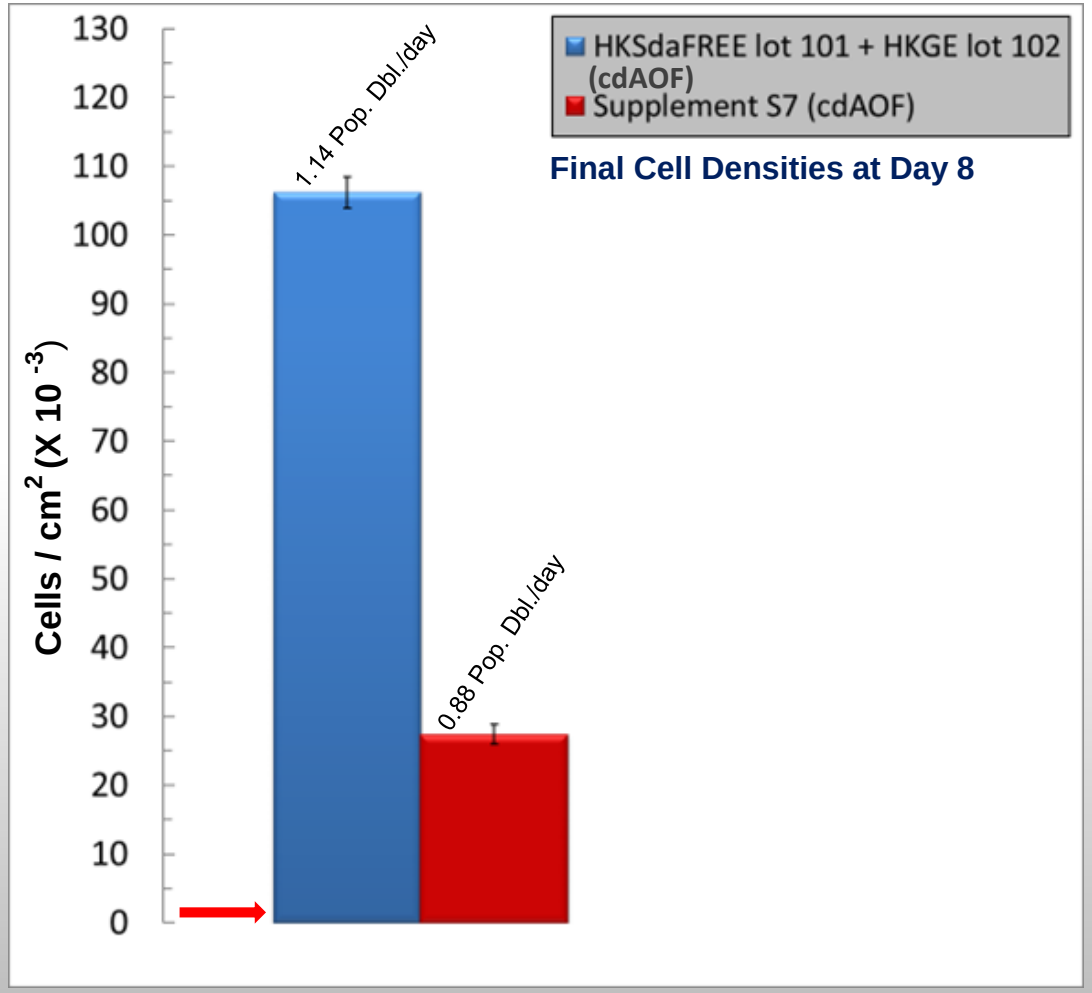
Post-primary Passage 4,
Day 23 Photomicrograph



Post-primary Passage 4,
Day 23 Photomicrograph

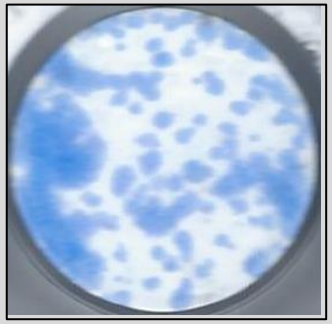
Methods: Chemically defined animal origin-free (cdAOF) adult human keratinocyte (HEKa) primary cell cultures were dissociated using TrypLE Select (Life Technologies) and plated into passage 1- 4 culture at 2,500 cells/cm^2, in triplicate in rh Collagen-1 coated 9.6 cm^2 wells. The HEKa were serially passaged into the cdAOF cell culture environment using AvantBio's HKSdaFREE KIT supplements and EpiLife basal medium (Life Technologies). Mean cumulative population doublings were calculated at the end of each passge, from triplicate wells, +/- the SEM. EpiLife is a trademark of Life Technologies Corporation.

Propagation of Neonatal Human Epidermal Keratinocytes (HEKn) Under AvantBio's Chemically-Defined, Animal Origin-free (cdAOF) Cell Culture Conditions: Comparison of HKSdaFREE KIT Supplements to Supplement S7.



Day 8 Stained Cells

HKSdaFREE lot 101 + HKGE lot 102 (cdAOF)

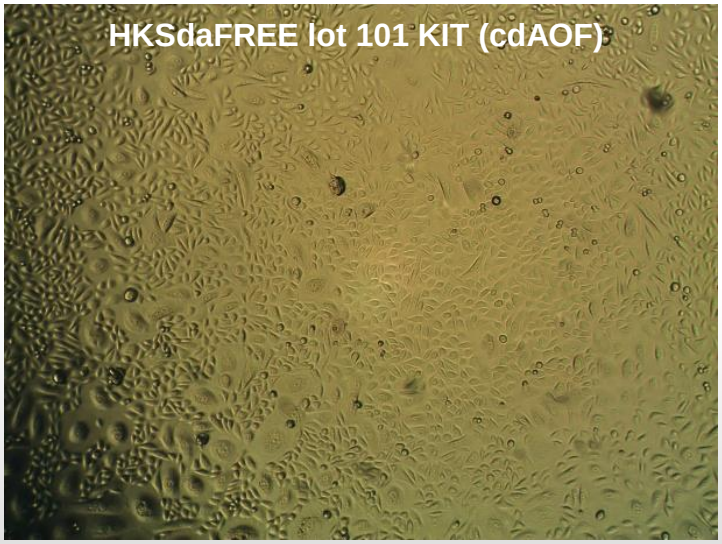


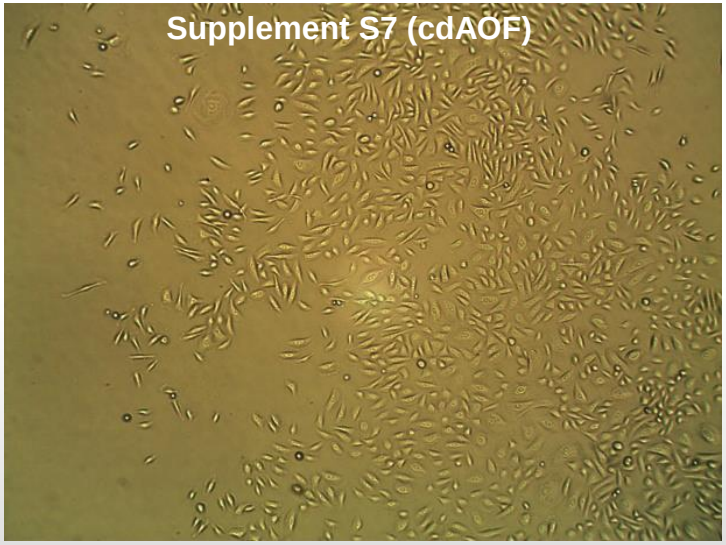
Supplement S7 (cdAOF)

Methods: Post-primary passage 2 human Neonatal Human Epidermal Keratinocytes (HEKn) were cultured under chemically-defined animal origin-free (cdAOF) conditions using EpiLife medium supplemented with either HKSdaFREE KIT supplements or Supplement S7, as indicated in the figure. Cells were plated in triplicate, into 6 well plates **without** human rec. hu. collagen-1 pre-coating, at low seeding density (250 viable cells/cm^2; see red arrow). Results are displayed as final mean cell densities after 8 days of culture, +/- the SEM. Pop. Dbl./day = average population doublings per day (a measure of relative growth rate). EpiLife Medium and supplement S7 are registered trademarks of Life Technologies Corporation.

Propagation of Neonatal Human Epidermal Keratinocytes (HEKn) Under AvantBio's Chemically-Defined, Animal Origin-free (cdAOF) Cell Culture Conditions: Comparison of HKSdaFREE KIT Supplements to Supplement S7.

Day 8 Photomicrographs



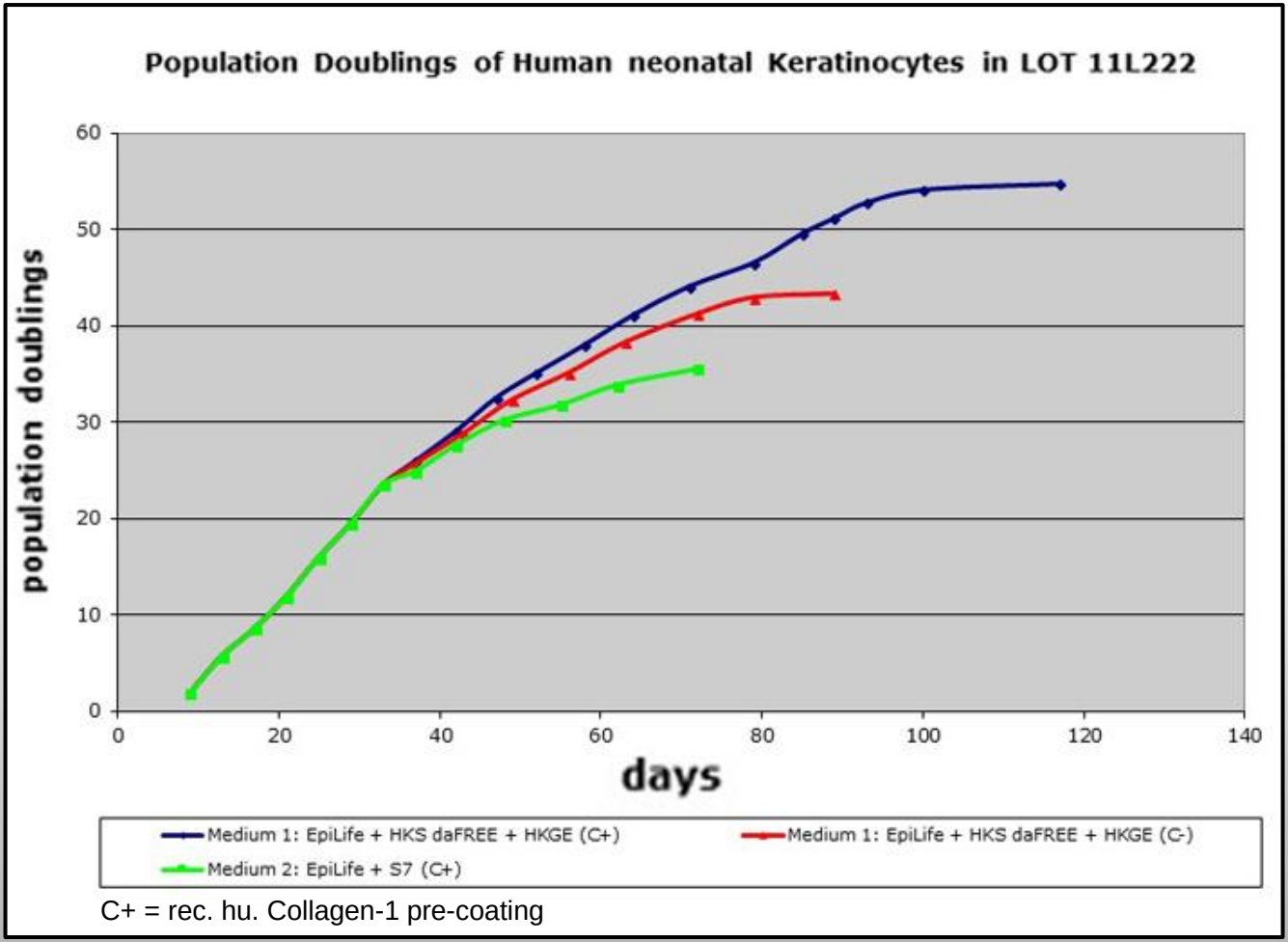


Methods: Post-primary passage 2 human Neonatal Human Epidermal Keratinocytes (HEKn) were cultured under chemically-defined animal origin-free (cdAOF) conditions using EpiLife medium supplemented with either HKSdaFREE KIT supplements or Supplement S7, as indicated in the figure. Cells were plated in triplicate, into 6 well plates **without** human rec. hu. collagen-1 pre-coating, at low seeding density (250 viable cells/cm^2). Results are displayed as representative photomicrographs at day 8. EpiLife Medium and supplement S7 are registered trademarks of Life Technologies Corporation.

CULTURING GROWTH RATE IMPROVEMENT OF NEONATAL FORESKIN KERATINOCYTES WITH A NEW DEFINED ANIMAL ORIGIN-FREE CELL CULTURE MEDIUM SUPPLEMENT

Gunther Verween, Jean-Pierre Draye, Haaike Colemonts-Vroninks, Gilbert Verbeken, Thomas Rose, Serge Jennes, Jean-Paul Pirnay Human Cell and Tissue Banks, Laboratory for Molecular and Cellular Technology, Burn Wound Centre, Queen Astrid Military Hospital, Brussels

From September 2012 "Wound Repair and Regeneration 20(5):A118-A118



C+ = rec. hu. Collagen-1 pre-coating

Beta Site Study Independently Demonstrates That HKSdaFREE KIT Supplements show improved performance Over Thermofisher's cdAOF Supplement S7 in Preclinical Testing.

Independently Confirms That When Using HKSdaFREE KIT Supplements, Pre-coating the Cell Culture Surface With rec. hu. Collagen-1 (c+) is Not Required for Efficient Post-primary Serial Propagation

Reconstructed Human Epidermis (RHE)

Potential Applications Under cdAOF Conditions

For In Vitro Cutaneous (Skin) Testing

- ***Irritancy***
- ***Corrosion***
- ***Genotoxicity***
- ***Permeation***
- ***Other***

For Cell-based and Engineered Tissue-based Cutaneous (Skin) Therapies:

- ***Replacement***
- ***Regeneration***
- ***Repair***

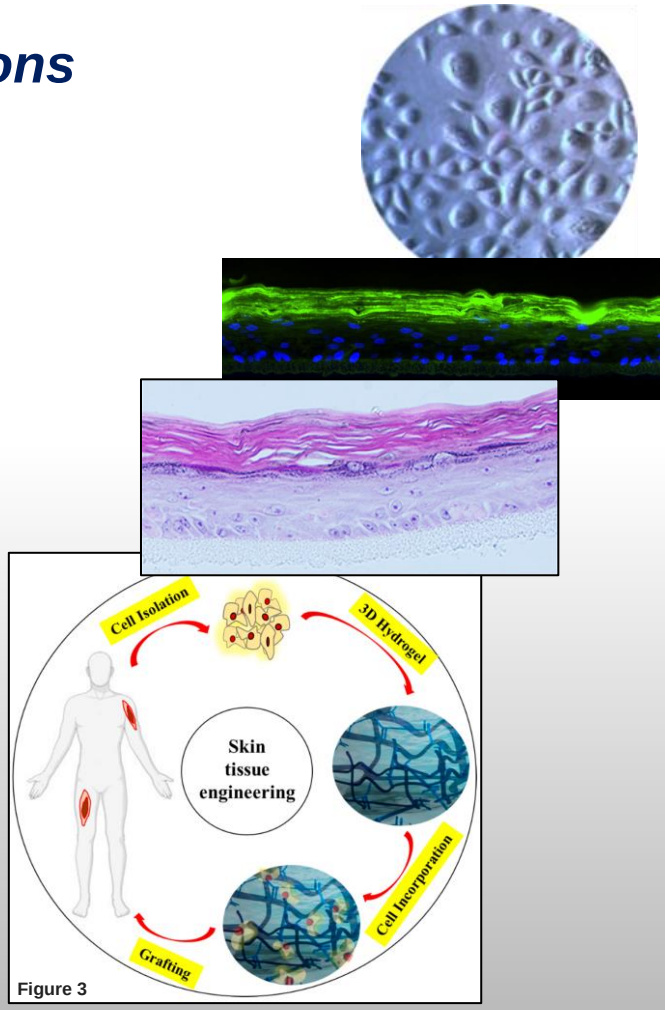


Figure 3

Appl. Sci. **2021**,*11*(4), 1493 https://www.mdpi.com/openaccess

The HKSdaFREE KIT Supplement System Supports Reconstructed Human Epidermis (RHE) from Neonatal Human Epidermal Keratinocytes (HEKn) Cultured on Polycarbonate Filters, Under Chemically-defined Animal Origin-Free (cdAOF) Conditions.

From J. Invest. Derm. Vol 132 supplement 1, p. S147, 2012 Society for Investigative Dermatology (SID) Annual Meeting & May 9 - 12, 2012, Raleigh, NC

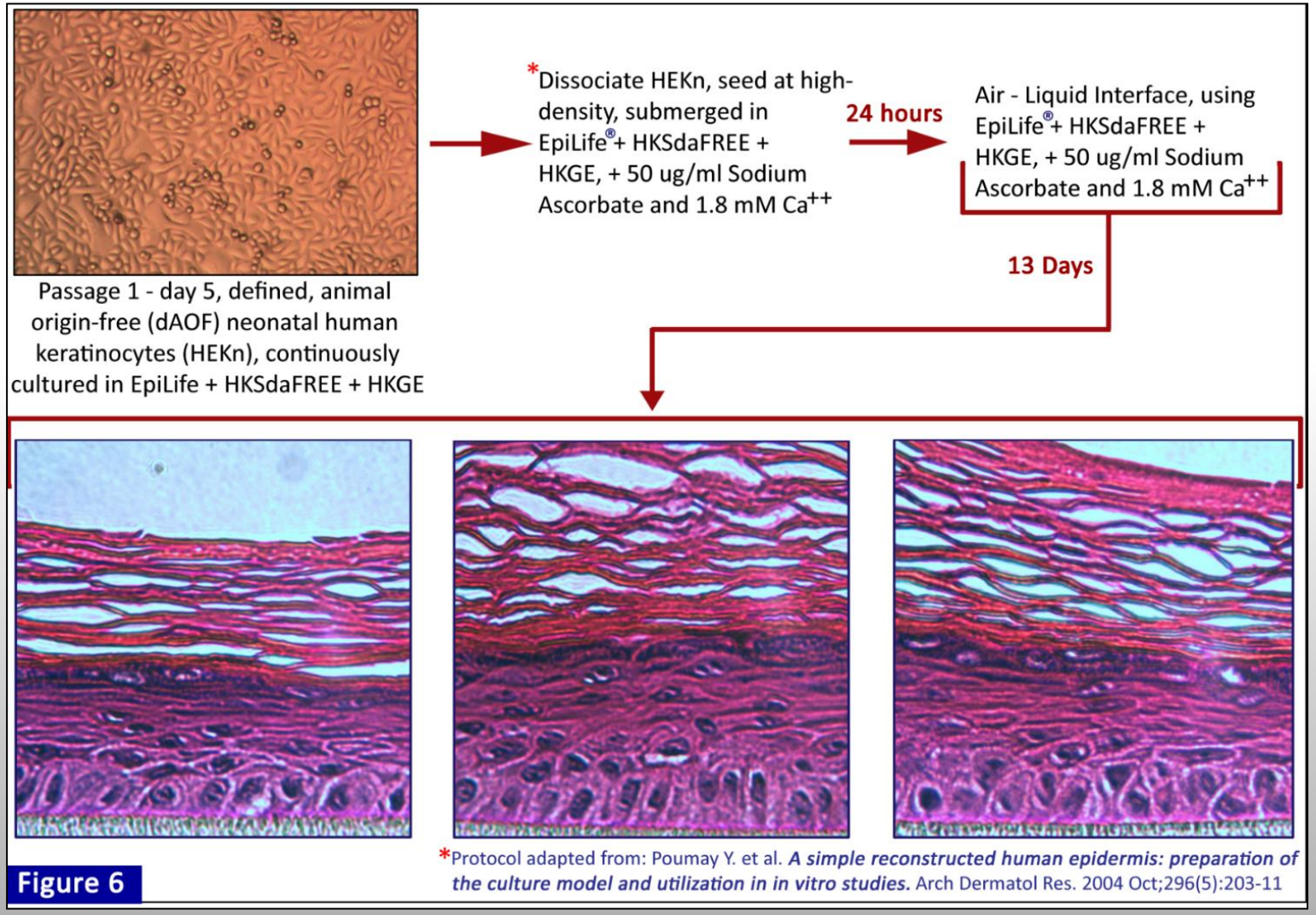


Figure 6

EpiLife Medium is a trademark of Life Technologies Corporation.

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RESEARCH ARTICLE

BIOENGINEERING & TRANSLATIONAL MEDICINE

3D bioprinting of an implantable xeno-free vascularized human skin graft

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Abstract

Bioengineered tissues or organs produced using matrix proteins or components derived from xenogeneic sources pose risks of allergic responses, immune rejection, or even autoimmunity. Here, we report successful xeno-free isolation, expansion, and cryopreservation of human endothelial cells (EC), fibroblasts (FBs), pericytes (PCs), and keratinocytes (KCs). We further demonstrate the bioprinting of a human skin substitute with a dermal layer containing xeno-free cultured human EC, FBs, and PCs in a xeno-free bioink containing human collagen type I and fibronectin layered in a biocompatible polyglycolic acid mesh and subsequently seeded with xeno-free human KCs to form an epidermal layer. Following implantation of such bilayered skin grafts on the dorsum of immunodeficient mice, KCs form a mature stratified epidermis with rete ridge-like structures. The ECs and PCs form human EC-lined perfused microvessels within 2 weeks after implantation, preventing graft necrosis, and eliciting further perfusion of the graft by angiogenic host microvessels. As proof-of-concept, we generated 12 individual grafts using a single donor of all four cell types. In summary, we describe the fabrication of a bioprinted vascularized bilayered skin substitute under completely xeno-free culture conditions demonstrating feasibility of a xeno-free approach to complex tissue engineering.

KEYWORDS
3D bioprinted, skin, vascularized, xeno-free

AVANTBIO™

Human Epidermal Keratinocytes Isolated and Propagated in EpiLife Basal Medium, Plus AvantBio's Chemically-defined Animal Origin-free **HKSdaFREE KIT** Supplements, Were Utilized as a Novel Bioink to Generate an Implantable, 3D Bioprinted Skin Equivalent.

EpiLife Medium is a trademark of Life Technologies Corporation.

BIOENGINEERING & TRANSLATIONAL MEDICINE
Bioeng Transl Med. 2022;e10324.
https://doi.org/10.1002/btm2.10324

3D bioprinting of an implantable xeno-free vascularized human skin graft

Tania Baltazar[1] | Bo Jiang[2,3] | Alejandra Moncayo[4,5] | Jonathan Merola[2,6] | Mohammad Z. Albanna[7,8] | W. Mark Saltzman[9] | Jordan S. Pober[1]

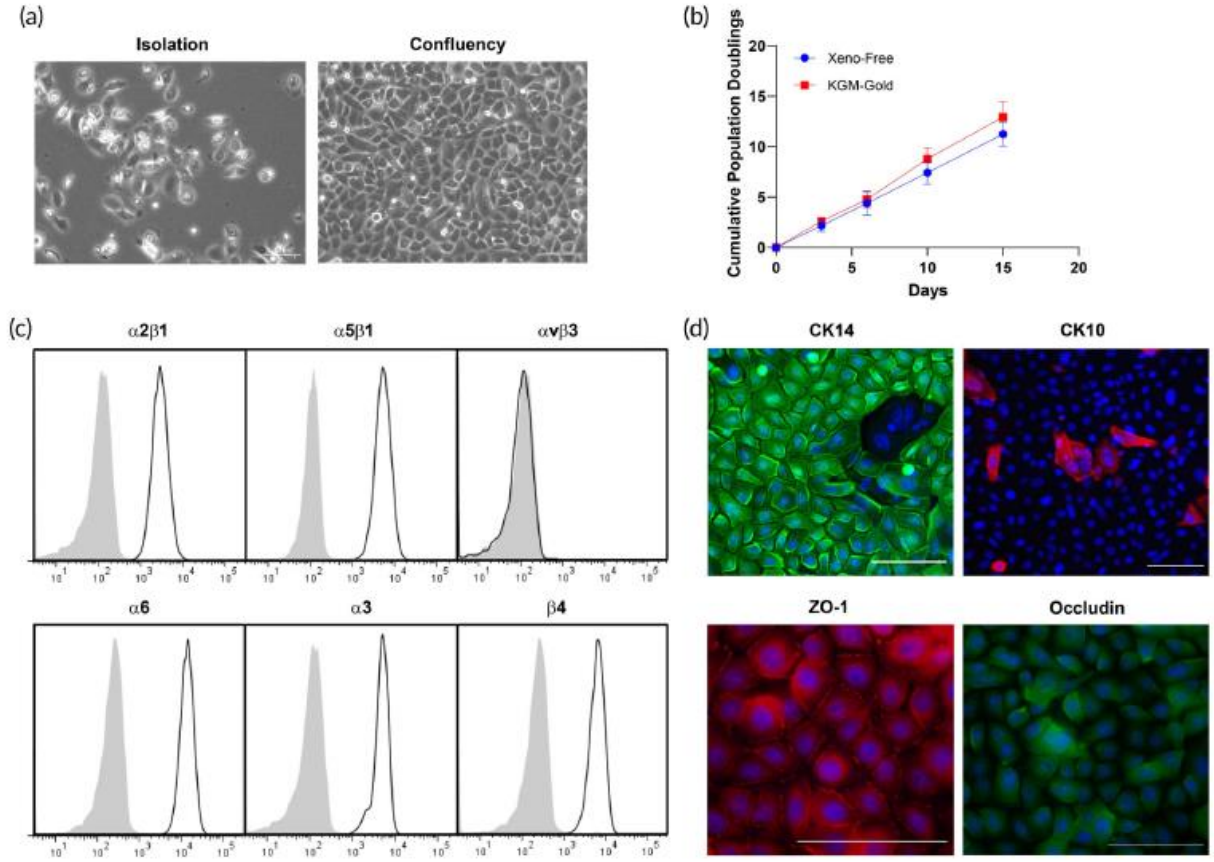


FIGURE 5 Phenotyping characterization of human keratinocytes under xeno-free conditions. (a) Live phasecontrast microscopy images of keratinocytes after isolation from the epidermis of donor foreskin and at the confluency state. (b) The cumulative population doublings of keratinocytes cultured under xeno-free conditions is comparable to KGM-Gold medium. (c) Flow cytometry analysis confirmed the expression of integrins α2β1, α5β1, α6, α3, and β4, but not αvβ3. (d) Confocal microscopy exhibiting CK14, CK10, junctional ZO-1, and intracellular occludin staining. Scale bar = 100 μm. Representative of three independent donors

Phenotypic Characterization of Human Epidermal Keratinocytes Isolated and Propagated in EpiLife Basal Medium Plus AvantBio's Chemically-defined Animal Origin-free **HKSdaFREE + KIT** Supplements.

BIOENGINEERING & TRANSLATIONAL MEDICINE
Bioeng Transl Med. 2022;e10324.
https://doi.org/10.1002/btm2.10324

3D bioprinting of an implantable xeno-free vascularized human skin graft

Tania Baltazar[1] | Bo Jiang[2,3] | Alejandra Moncayo[4,5] | Jonathan Merola[2,6] | Mohammad Z. Albanna[7,8] | W. Mark Saltzman[9] | Jordan S. Pober[1]

Successful Implantation of Full-thickness, Engineered 3D Bioprinted Skin Equivalents Into SCID/bg Mice Within 8 Days

Human Epidermal Keratinocytes Isolated and Propagated in AvantBio's Chemically-defined Animal Origin-free (cdAOF) **HKSdaFREE KIT** Supplements, in EpiLife Basal Medium.

Switch to cdAOF **HKSdaFREE KIT** Supplements in EpiLife Basal Medium, Plus Elevated Calcium, rh KGF and Ascorbic Acid. Apply as **Novel Epidermal Cell Bioink** on Day 4 for the Bioprinting of The Differentiated 3D Epidermis.

Dermal Bioink:
Cells, Human Serum, Human Fibronectin, F12 Basal Medium, Human Collagen-1, Buffer and VitroGel Hydrogel Matrix

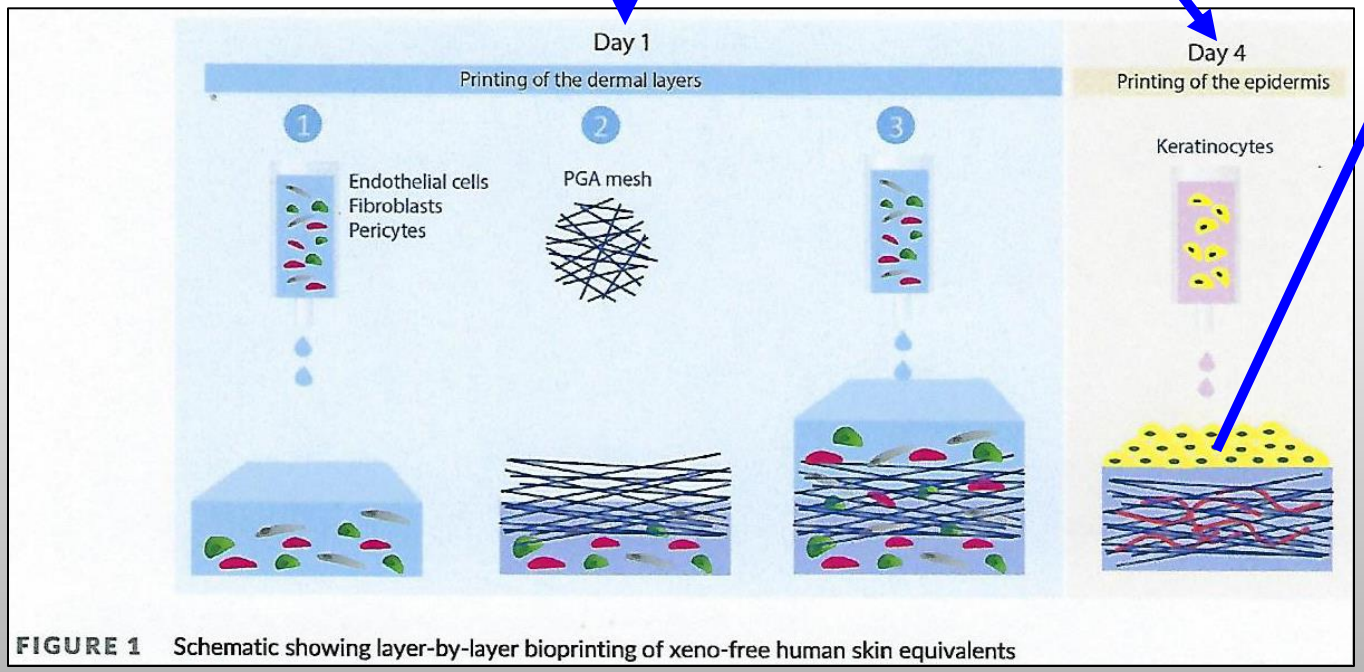
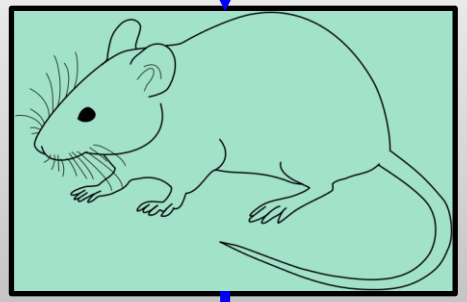


FIGURE 1 Schematic showing layer-by-layer bioprinting of xeno-free human skin equivalents

24 Hours Post-epidermal bioprinting, transfer 3D Bioprinted Tissue to 6-well plates and culture in cdAOF **HKSdaFREE KIT** Supplements, in EpiLife Basal Medium, Plus Elevated Calcium, rh KGF and Ascorbic Acid for 3 Additional Days.

Engraftment of 3D Bioprinted Skin Equivalent Into SCID/bg Mice



Obtain Biopsies From 3D-bioprinted Skin Equivalent Implants at 2-6 Weeks for Analysis.

BIOENGINEERING & TRANSLATIONAL MEDICINE
Bioeng Transl Med. 2022;e10324.
https://doi.org/10.1002/btm2.10324

3D bioprinting of an implantable xeno-free vascularized human skin graft

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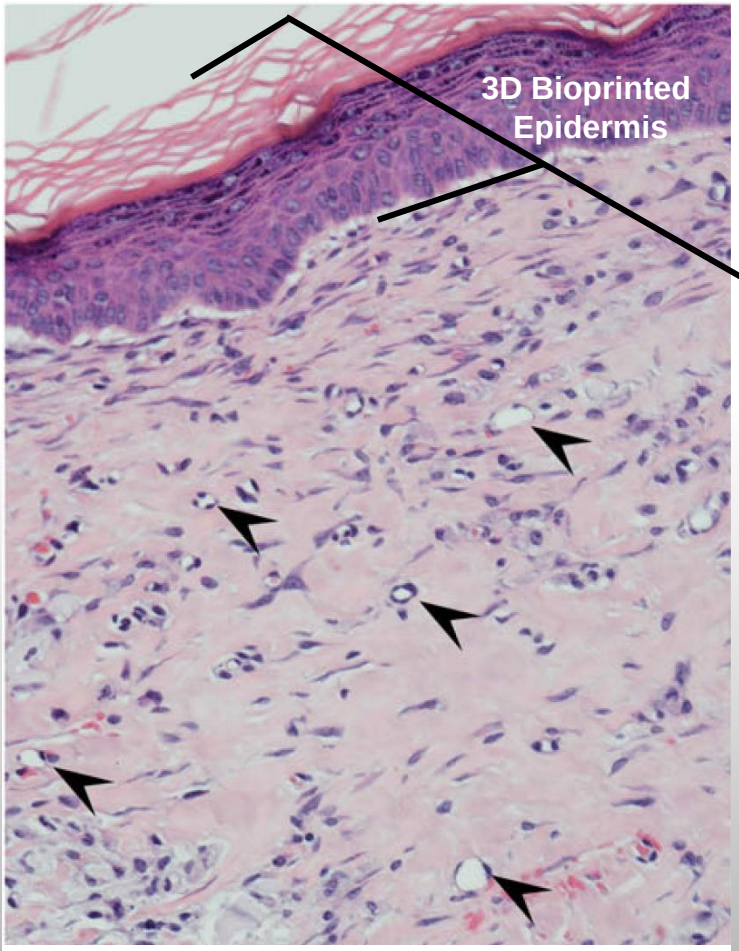
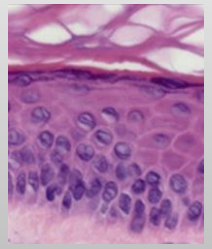


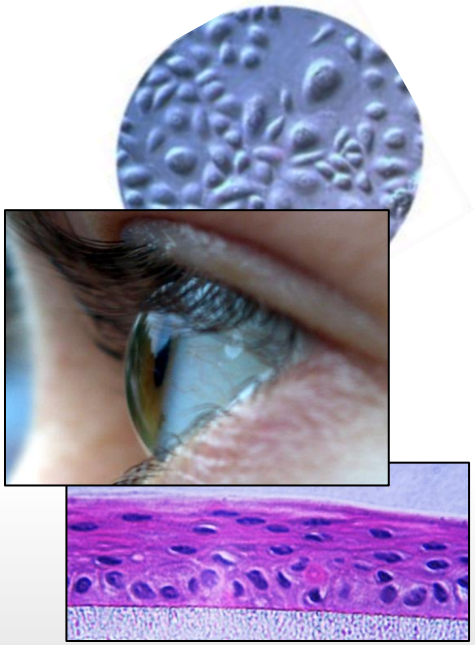
Figure 7(b): Histology at 4 weeks Post-engraftment



Normal Human Epidermis (H&E Stained) Histology

Human Epidermal Keratinocytes Isolated and Propagated in EpiLife Basal Medium Plus AvantBio's Chemically-defined Animal Origin-free **HKSdaFREE + HKGE KIT** Supplements Were Utilized as a **Novel Epidermal Cell Bioink** to Generate a 3D Bioprinted Skin Equivalents, Implanted Into SCID/bg Mice (at 4 weeks).

Human Corneal Epithelial Cells (HCEC)



Potential Applications in Basic and Preclinical Laboratory Research

- ***2D Corneal Epithelial Cell Cultures***
  - ***Human Corneal Epithelial Cells***
- ***3D Bioprinted, Automated or Manually Reconstructed Tissue, Using Corneal Epithelial Cells***
  - ***3D Reconstructed Corneal Epithelium***
  - ***3D Reconstructed Corneal Epithelium on a Chip***
  - ***3D Reconstructed Corneal Epithelium for In Vitro Testing (e.g., Irritancy, Bacterial Adhesion, Omics, Other)***

Potential Clinical Applications in Regenerative and Esthetic Medicine Using Corneal Epithelial Cell Cultures

- ***2D Cell-based Corneal Therapies***
- ***3D Reconstructed Tissue-based Corneal Therapies***

A NEW CELL CULTURE SYSTEM FOR THE DEFINED, ANIMAL ORIGIN-FREE PRIMARY ISOLATION AND SERIAL PROPAGATION OF ADULT HUMAN CORNEAL EPITHELIAL CELLS AND ADULT HUMAN CORNEAL FIBROBLASTS

Paul Cook, Rolf Winter, Jeffrey Brown, AvantBio Corporation, Vancouver WA USA

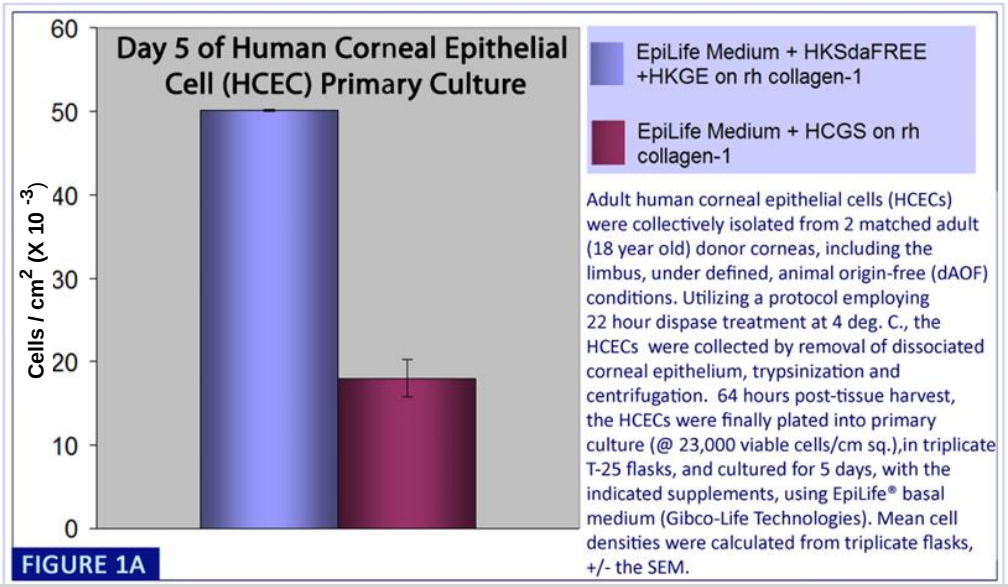
Abstracts 22nd Annual Meeting of the European Tissue Repair Society, Athens, Greece, October 4–5, 2012

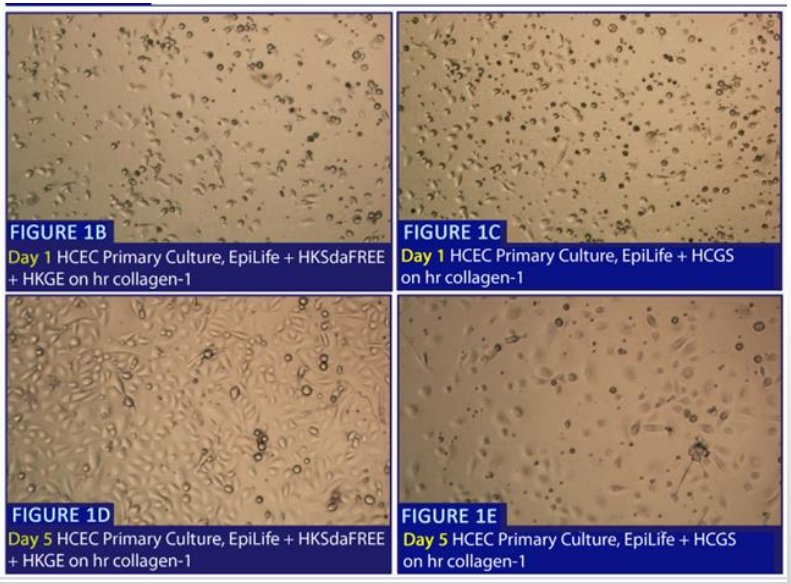
In Wound Rep. and Reg., Vol. 20 (5) p. A89, 2012

Animal-derived components have been traditionally used as supplements to propagate normal human corneal cells and frequently include human- or bovine-derived serum, bovine pituitary extract (BPE), bovine serum-derived albumin (BSA) or replication-incompetent rodent feeder-layer cells. Defined, animal origin-free (dAOF) cell culture can reduce or eliminate the risk of transmitting human pathogens or xenopathogens from contaminated animal-derived cell culture components to humans undergoing cell- or engineered tissue-based therapies that employ the use of cultured human corneal epithelial cells (HCEC) or human cornea-derived fibroblasts (keratocytes). We have developed a new dAOF cell culture supplement system **(HKSdaFREE + HKGE)** that promotes the primary isolation and serial propagation of adult HCEC in a complete dAOF cell culture environment, using commercially available human keratinocyte basal media (e.g. EpiLife from Life Technologies). For adult HCEC (18-year old donor) cultured in EpiLife basal medium and HKSdaFREE + HKGE, 47 and 70 cumulative post-primary population doublings can be attained on untreated and recombinant human collagen-1 pretreated substrata, respectively. Early passage growth rates exceed 1 population doubling/ day and serial propagation of HCEC is not dependent on precoating the cell culture surface. Compared to previous animal component-containing HCEC cell culture systems, our new dAOF HCEC cell culture system performance either exceeds (e.g. low-calcium, BPE), or compares favorably (e.g. bovine or human serum, high-calcium, feeder cells), to the predecessor HCEC culture systems. **Our new dAOF HCEC culture system also supports the generation of histologically correct stratified human corneal epithelium under complete dAOF culture conditions.** Using a variant dAOF supplement system (HFSdaFREE + HFGE + HFGE2), recent results also demonstrate the efficient primary and post-primary propagation of human keratocytes. Collectively, our new dAOF cell culture system supplements may represent a breakthrough in the dAOF culture of human corneal-derived cells for potential use in cell-based or engineered tissue-based therapy of damaged or diseased human corneas.

AvantBio's HKSdaFREE KIT Supplements Improve the Isolation and Propagation of Primary Adult Human Corneal Epithelial Cells (HCEC) Under Chemically Defined Animal Origin-free (cdAOF) Conditions.

From Abstracts 22nd Annual Meeting of the European Tissue Repair Society, Athens, Greece, October 4–5, 2012 In Wound Rep. and Reg., Vol. 20 (5) p. A89, 2012

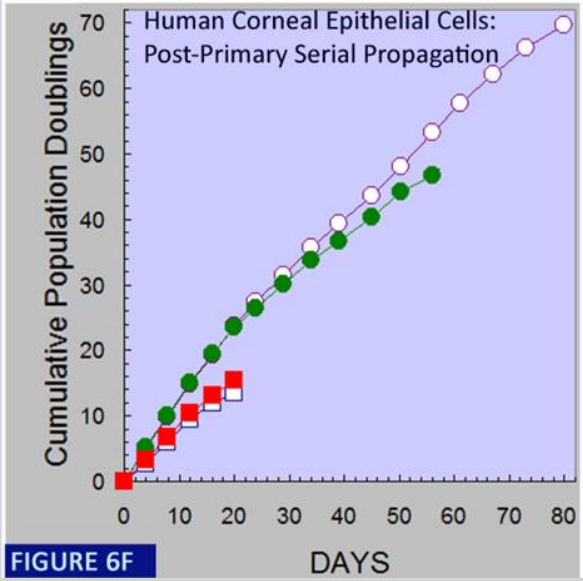


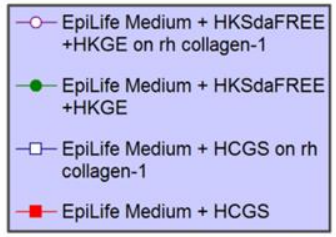


EpiLife and HCGS are trademarks of Life Technologies Corporation. HCGS is comprised of Bovine Pituitary Extract and growth factors.

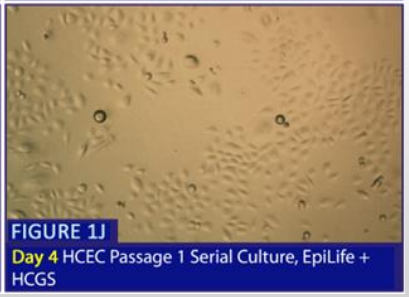
AvantBio's HKSdaFREE KIT Supplements Improve the Post-primary Serial Propagation of Adult Human Corneal Epithelial Cells (HCECs) Under Chemically Defined Animal Origin-free (cdAOF) Conditions. Efficient Serial Propagation is not Dependent on Pre-coating the Cell Culture Surface with Recombinant Human Collagen-1 (rh Collagen-1).

From Abstracts 22nd Annual Meeting of the European Tissue Repair Society, Athens, Greece, October 4–5, 2012 In Wound Rep. and Reg., Vol. 20 (5) p. A89, 2012





Defined, animal origin-free (dAOF) adult primary HCEC cell cultures (as shown above in figures 6A-E) were dissociated under dAOF conditions and plated into passage 1-16 subculture (at 2,500 viable cells/cm sq.), in duplicate T-25 flasks. The HCECs were serially passaged in the dAOF cell culture environment using the indicated dAOF supplements and EpiLife® basal medium (Gibco-Life Technologies). Values represent mean cumulative population doublings, +/- SEM.



EpiLife and HCGS are trademarks of Life Technologies Corporation. HCGS is comprised of Bovine Pituitary Extract and growth factors.

Reconstructed Human Corneal Epithelium (RHCE)

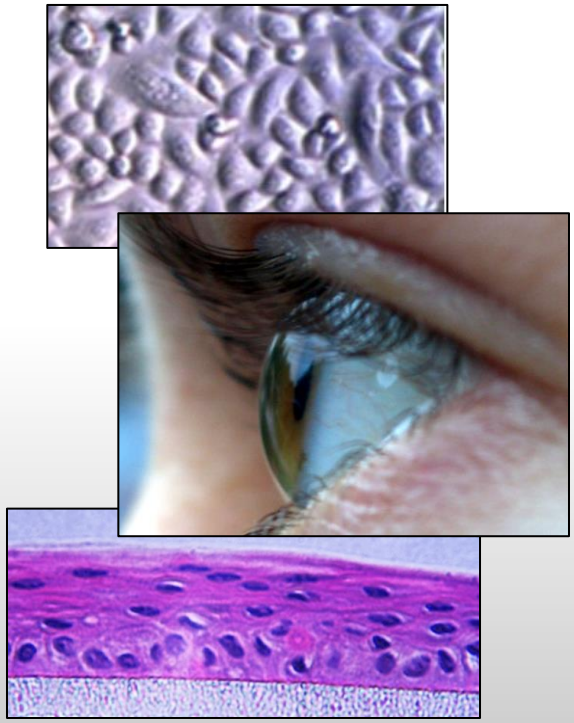
Potential Applications Under cdAOF Conditions

For In Vitro Ocular (Cornea) Testing:

- ***Irritancy***
- ***Bacterial Adhesion***
- ***Omics***
- ***Other***

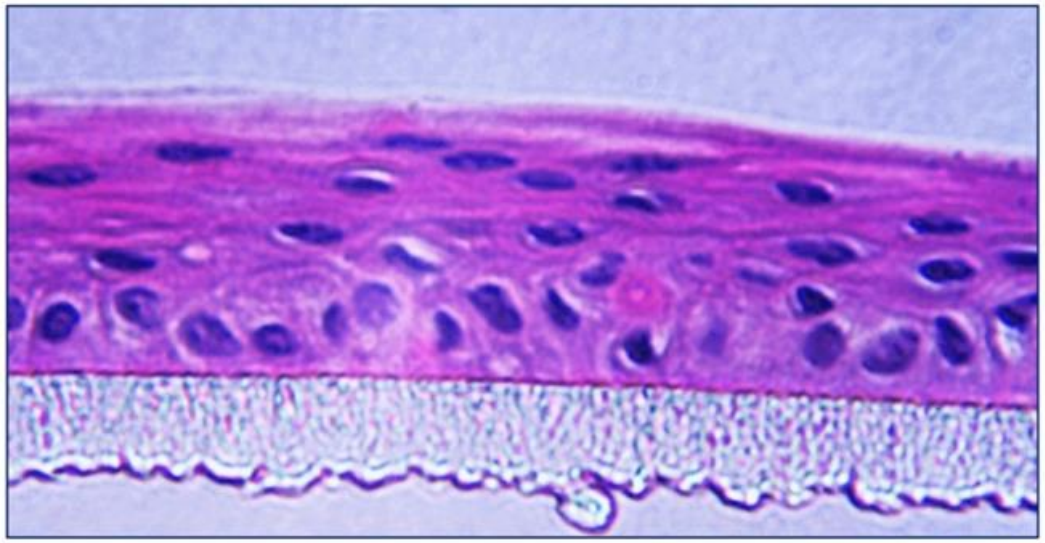
For Cell-based and Engineered Tissue-based Ocular (Cornea) Therapies:

- ***Replacement***
- ***Regeneration***
- ***Repair***



The HKSdaFREE KIT Supplement System Supports the Reconstruction of Human Corneal Epithelium (RHCE) Under Chemically-defined Animal Origin-free (cdAOF) Culture Conditions

From Abstracts 22nd Annual Meeting of the European Tissue Repair Society, Athens, Greece, October 4–5, 2012 In Wound Rep. and Reg., Vol. 20 (5) p. A89, 2012



Formalin Fixation, H&E Staining. Digital Photomicrograh, w. 40 x objective (non-phase) plus digital magnification (apx. 10x). Copyright 2012, AvantBio Corp. All Rights Reserved.

Passage 1 human corneal epithelial cells (HCEC) were plated and propagated on recombinant human collagen 1 as described for figure 5, dissociated, and then plated at high-density in 0.63 sq. cm Millicell polycarbonate filter inserts (Millipore), under dAOf cell culture conditions. The dAOf HCEC cultures were then stratified using both submerged and near air-liquid interface dAOF cell culture conditions, for a combined total of 16 days in dAOF cell culture.

EpiLife (cdAOF media) is a trademark of Life Technologies Corporation.

Bio-compatible Engineered Silk-derived Fibroin Protein Can Be Fabricated Into a Variety Configurations, in Some cases for Applications in Regenerative Medicine, That Include the Delivery of Therapeutic Cells or Tissue on Discs (Mats), Scaffolds (Fibers) and Hydrogels

International Journal of *Molecular Sciences*

MDPI

Review

Silk Fibroin as a Functional Biomaterial for Tissue Engineering

Weizhen Sun [1], David Alexander Gregory [1,2], Mhd Anas Tomeh [1] and Xiubo Zhao [1,3,*]

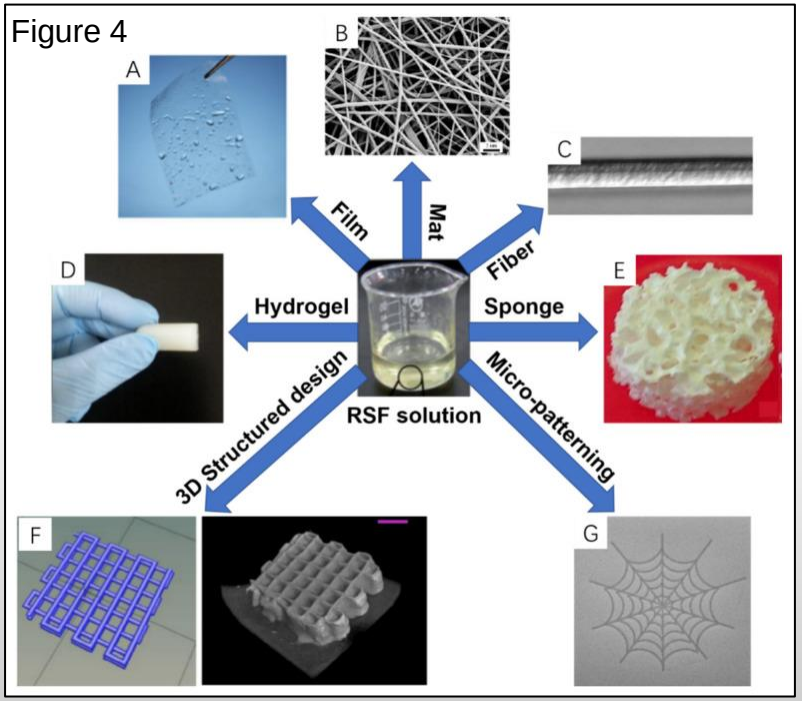
Int. J. Mol. Sci. **2021**, *22*, 1499. https://doi.org/10.3390/ijms22031499

Human corneal epithelial equivalents constructed on *Bombyx mori* silk fibroin membranes

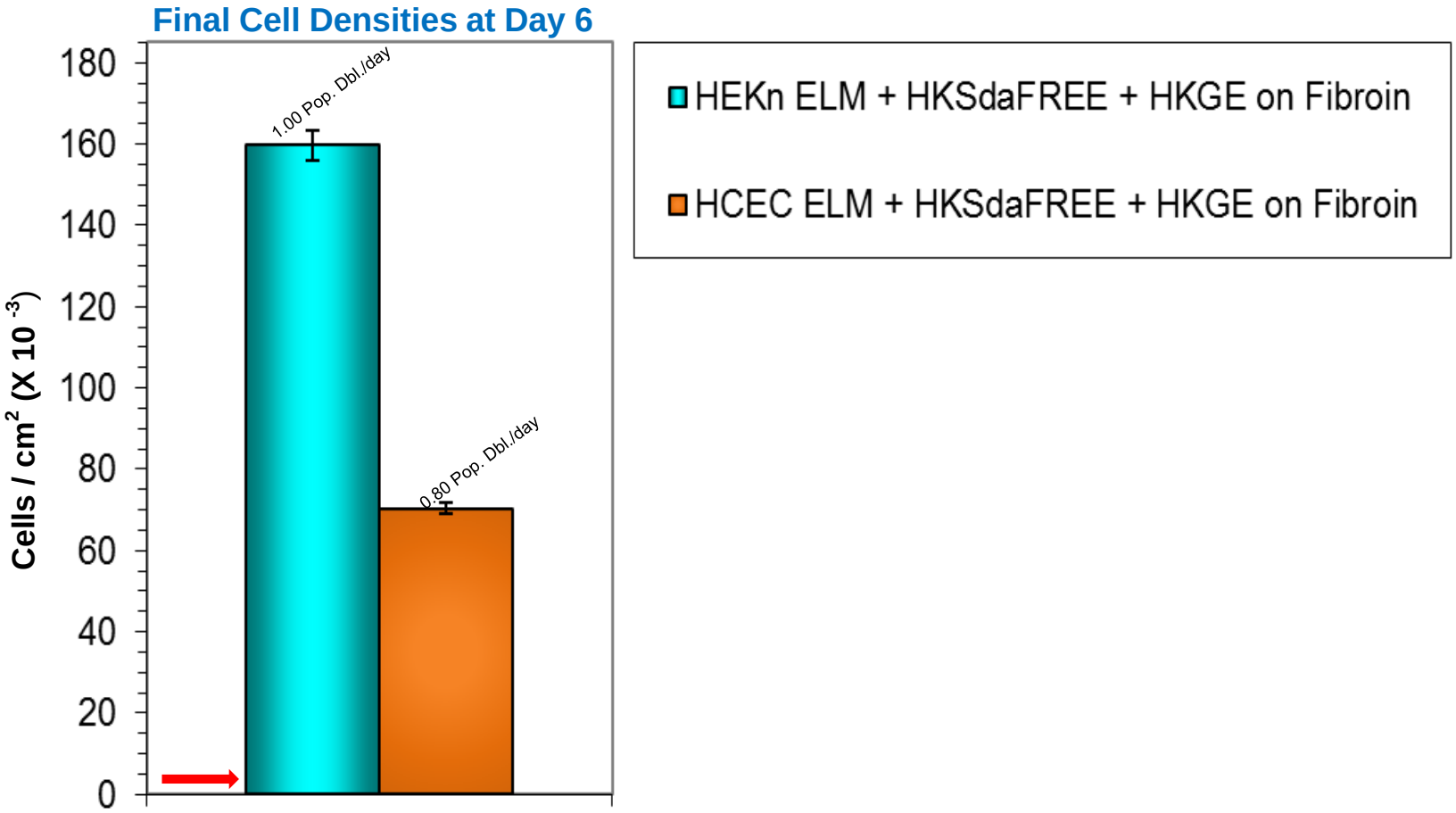
Laura J. Bray [a,b,c], Karina A. George [a,b,c], S. Louise Ainscough [a,b,c], Dietmar W. Hutmacher [c,d], Traian V. Chirila [a,e,f,g], Damien G. Harkin [a,b,c,*]

Biomaterials 32 (2011) 5086–5091

Figure 4



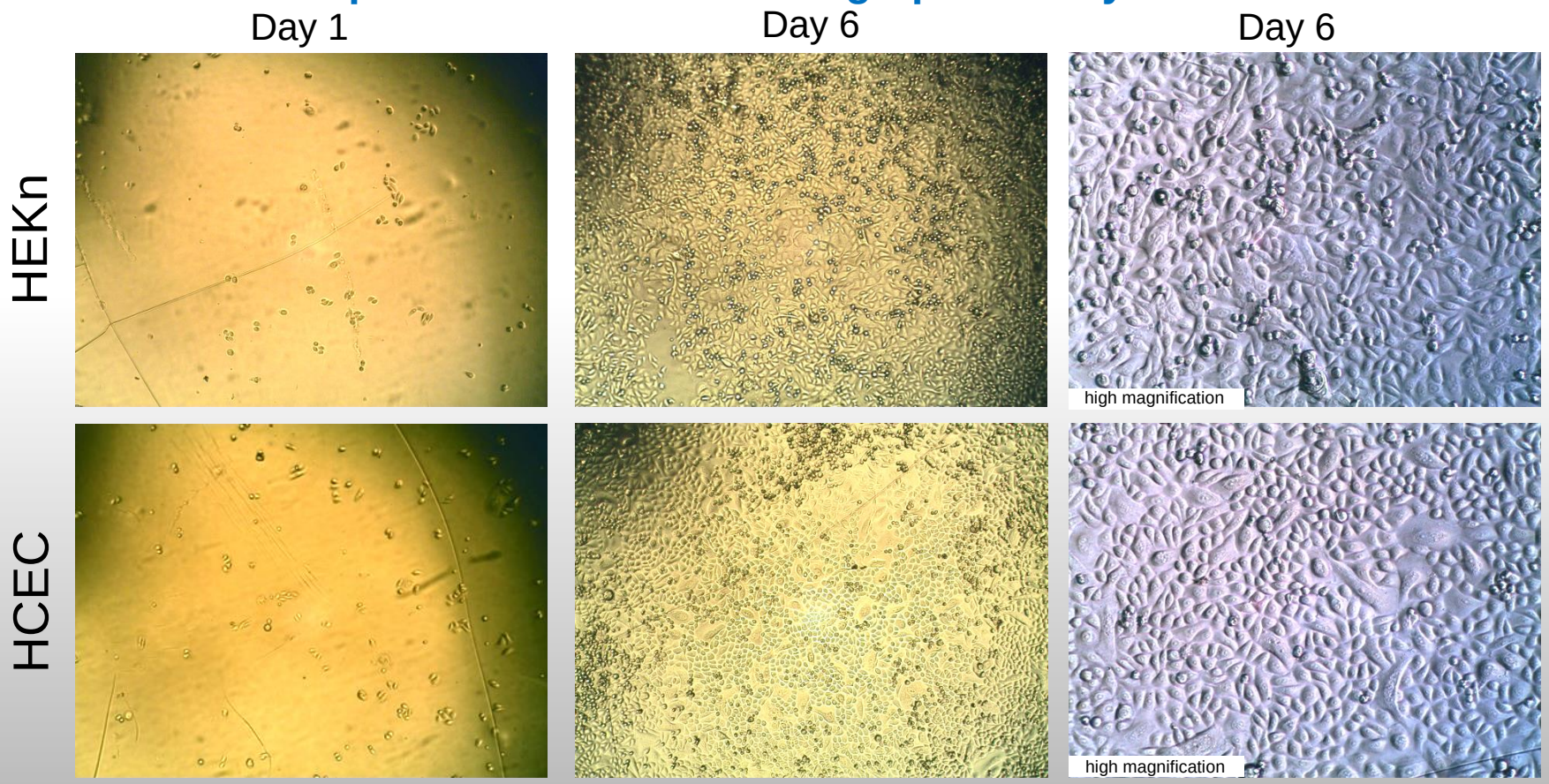
Propagation of Neonatal Human Epidermal Keratinocytes (HEKn) and Adult Human Corneal Epithelial Cells (HCEC) on Bio-compatible, Silk-derived Fibroin Discs (Mats). Cells Cultured Under Chemically-defined Animal Origin-free (cdAOF) Cell Culture Conditions, Using AvantBio's HKSdaFREE KIT Supplements.



Methods: Post-primary passage 2 cdAOF human Neonatal Human Epidermal Keratinocytes (HEKn) and cdAOF Human Corneal Epithelial Cells (HCEC) were cultured under chemically-defined animal origin-free (cdAOF) conditions, using HKSdaFREE KIT supplements. Cells were cultured on silk-derived Fibroin discs (mats in 24-well format), in triplicate, without human rec. hu. collagen-1 pre-coating. Results are displayed as final mean cell densities after 6 days of culture, +/- the SEM. Seeding density = 2,500 cells/cm^2 (see red arrow). Pop. Dbl./day = average population doublings per day (a measure of relative growth rate). ELM = EpiLife Medium, a registered trademark of Life Technologies Corporation.

Propagation of Neonatal Human Epidermal Keratinocytes (HEKn) and Adult Human Corneal Epithelial Cells (HCEC) on Bio-compatible, Silk-derived Fibroin Discs (Mats). Cells Cultured Under Chemically-defined Animal Origin-free (cdAOF) Cell Culture Conditions, Using AvantBio's HKSdaFREE KIT Supplements

Representative Photomicrographs at Day 1 and 6



Methods: Post-primary passage 2 cdAOF human Neonatal Human Epidermal Keratinocytes (HEKn) and cdAOF Human Corneal Epithelial Cells (HCEC) were cultured under chemically-defined animal origin-free (cdAOF) conditions, using HKSdaFREE KIT supplements. Cells were cultured on silk-derived Fibroin discs (mats in 24-well format), in triplicate, without human rec. hu. collagen-1 pre-coating. Results are displayed as final cell densities after 6 days of culture, +/- the SEM. Seeding density = 2,500 cells/cm^2, EpiLife Medium, a registered trademark of Life Technologies Corporation.

Human Gingival Epithelial Cells (HGEC)

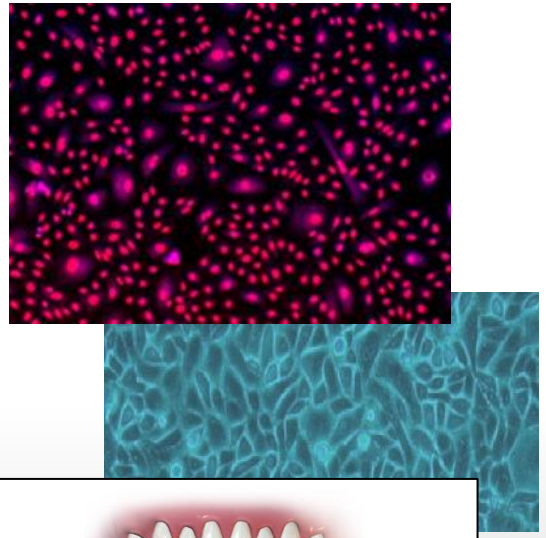
Potential Applications Under cdAOF Conditions:

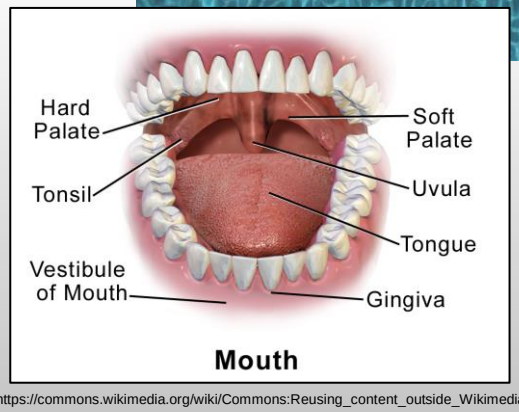
For Basic Experimental & Preclinical Laboratory Gingival (Oral) Research:

- ***2D HGEC Cultures***
- ***3D Bioprinted, Automated or Manually Reconstructed Gingival Epithelium***
- ***3D Partial- and Full-Thickness Reconstructed Gingiva Equivalents in Therapeutic Animal Models***

For Future Regenerative Medicine Clinical Applications:

- ***HGEC Cell-based Gingival Therapies***
- ***3D Partial- and Full-Thickness Reconstructed Gingiva for Gingival Therapy***
  - ***Repair***
  - ***Regeneration***
  - ***Replacement***





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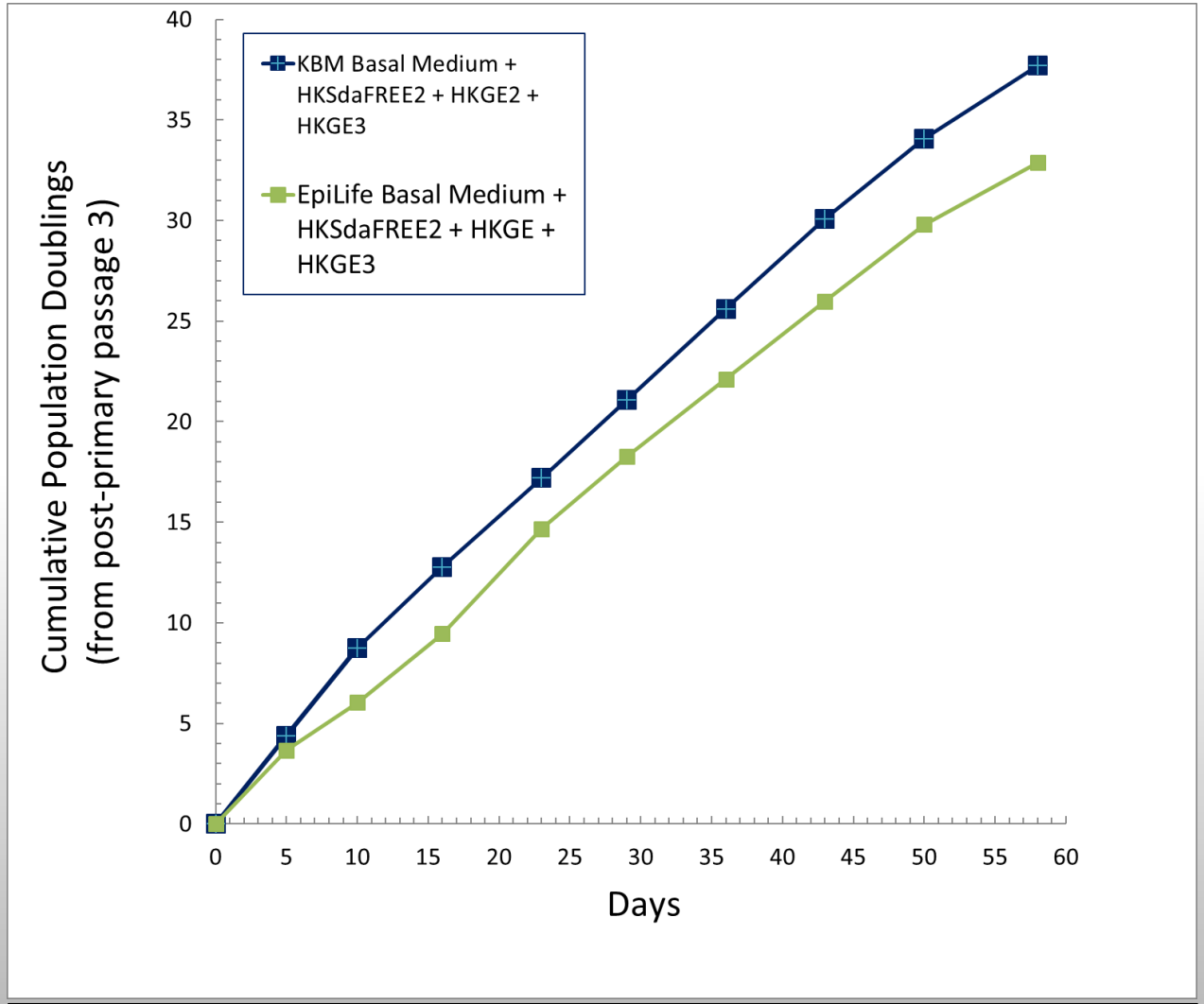
AvantBio's Second Generation Chemically Defined Animal Origin-free (cdAOF) HKSdaFREE2 KIT Supplements Efficiently Support the Post-primary Serial Propagation of Bovine Pituitary Extract (BPE)-Starved Human Gingival Epithelial Cells.



HKSdaFREE2 KIT
3-supplement component kit
(HKSdaFREE2, HKGE & HKGE3)

Methods: Passage 2, BPE-starved adult human gingival epithelial cells (from CELLnTEC, Bern Switzerland) were plated into passage 3 - 11 culture, plating at 2,500 viable cells/cm^2, in triplicate T-25 flasks. The cells were serially passaged in the chemically-defined, animal origin-free (cdAOF) cell culture environment using HKSdaFREE2 KIT supplements in EpiLife® basal medium (Life Technologies) or **calcium-free** KBM basal medium (LONZA). Values represent mean cumulative population doublings, +/- SEM. EpiLife Medium is a registered trademark of Life Technologies Corporation and KBM Medium is a trademark of LONZA corporation.

MDCK and Vero Immortalized Cell Lines

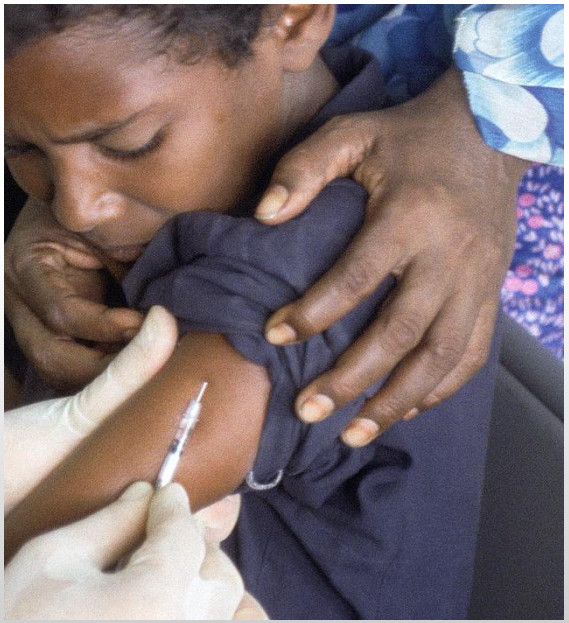
Other Potential Applications for AvantBio's First Generation Chemically-Defined Animal Origin-free (cdAOF) Cell Culture Technology Using HKSdaFREE: MDCK and Vero Bio-industrial Cell Lines
(historically used for vaccine and recombinant protein production)

Cell culture-derived flu vaccine: Present and future
Alberto Pérez Rubio and Jose María Eiros
Human Vaccine Immunotherapy. 2018; 14(8): 1874–1882.Published online 2018 Jun

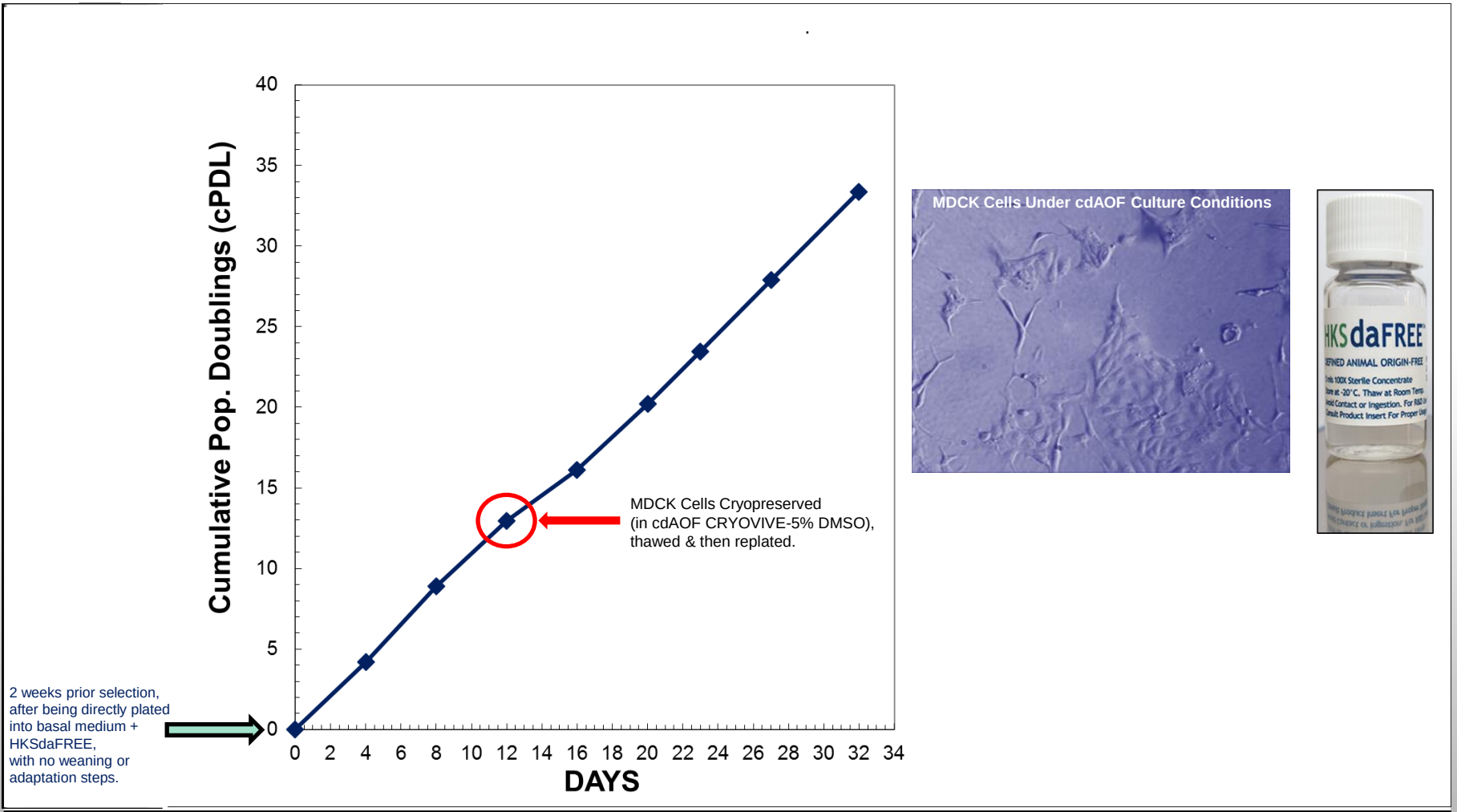
"Compared to other cell lines, the MDCK cells present several advantages for influenza vaccine production."

Vero cell upstream bioprocess development for the production of viral vectors and vaccines
Sascha Kiesslich and Amine A. Kamen
Biotechnol Adv. 2020 Nov 15; 44: 107608. Published online 2020 Aug

"The Vero cell line is considered the most used continuous cell line for the production of viral vectors and vaccines. Historically, it is the first cell line that was approved by the WHO for the production of human vaccines. Comprehensive experimental data on the production of many viruses using the Vero cell line can be found in the literature. However, the vast majority of these processes is relying on the microcarrier technology."

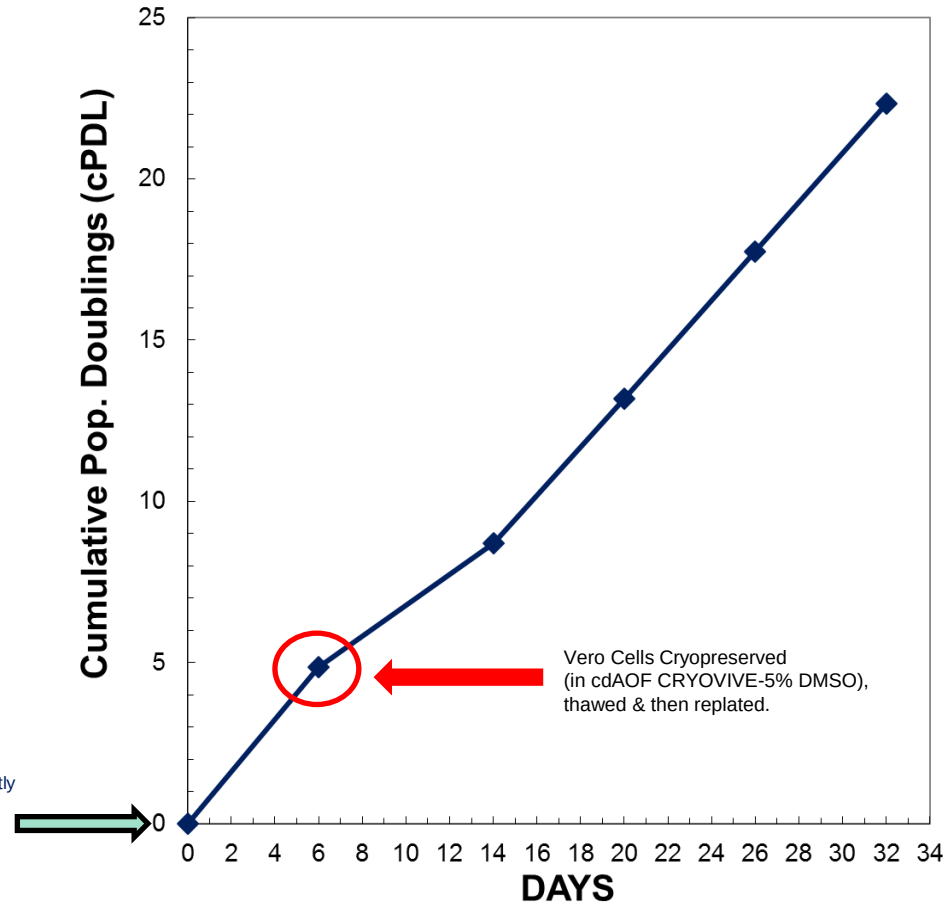


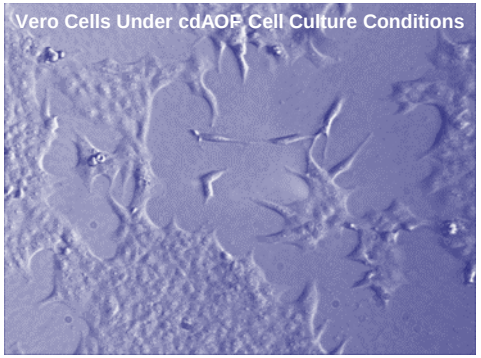
MDCK Immortalized Cell Line (NBL-2 ATCC CCL-34): Chemically-defined Animal Origin-free (cdAOF) Serial Propagation on Untreated Cell Culture Surfaces Using Basal Medium + HKSdaFREE



Methods: Passage 3**,** serum-starved MDCK cells (NGL-2 ATCC CCL-34) were plated into passage 3 - 8 culture (seeding at 2,500 viable cells/cm sq.), in triplicate wells (6-well plates). The cells were serially passaged in the chemically-defined, animal origin-free (cdAOF) environment using HKSdaFREE supplement in proprietary basal medium. Values represent mean cumulative population doublings, +/- SEM.

VERO Immortalized Cell Line (ATCC CCL-81): Chemically-defined Animal Origin-free (cdAOF) Serial Propagation on Untreated Cell Culture Surfaces Using Basal Medium + HKSdaFREE





Methods: Passage 3**,** serum-starved Vero cells (ATCC CCL-81) were plated into passage 3 - 8 culture (seeding at 2,500 viable cells/cm sq.), in triplicate wells (6-well plates). The cells were serially passaged in the chemically-defined, animal origin-free (cdAOF) environment using HKSdaFREE supplement in proprietary basal medium. Values represent mean cumulative population doublings, +/- SEM.

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