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(71) Applicant: **SMITH & NEPHEW, INC.** [US/US]; 1450
Brooks Road, Memphis, TN 38116 (US).

(72) Inventors: **JOVANOVIĆ, Aleksa**; c/o Smith & Nephew,
Inc., 1450 Brooks Road, Memphis, TN 38116 (US). **SHI,
Lei**; c/o Smith & Nephew, Inc., 1450 Brooks Road, Mem-
phis, TN 38116 (US). **LU, Yiling**; c/o Smith & Nephew,
Inc., 1450 Brooks Road, Memphis, TN 38116 (US). **RON-
FARD, Vincent**; c/o Smith & Nephew, Inc., 1450 Brooks
Road, Memphis, TN 38116 (US). **CAMPBELL, Jason**;
c/o Smith & Nephew, Inc., 1450 Brooks Road, Memphis,
TN 38116 (US). **COVARRUBIAS, Jacquelyn**; c/o Smith
& Nephew, Inc., 1450 Brooks Road, Memphis, TN 38116
(US). **RUFF, Annette**; c/o Smith & Nephew, Inc., 1450
Brooks Road, Memphis, TN 38116 (US). **MONTENEGRO,
Gladys**; c/o Smith & Nephew, Inc.,
1450 Brooks Road, Memphis, TN 38116 (US).

(74) Agent: **KRAWZSENEK, Michael, R.**; Norton Rose Ful-
bright US LLP, 98 San Jacinto Blvd., Suite 1100, Austin,
TX 78701 (US).

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(54) Title: BIOPRESERVATION MEDIUM AND USES FOR BIOPRESERVATION OF BIOLOGICAL MATERIALS

(57) Abstract: Biopreservation mediums containing poloxamers and cryoprotectants useful for all phases of biopreservation includ-
ing cryopreservation, thawing and hypothermic (cold storage) of biological materials such as cells, cell preparations, biological tis-
sues, and organs, plus their uses are disclosed. All phases of biopreservation can be conducted with one biopreservation medium.



DESCRIPTION

BIOPRESERVATION MEDIUM AND USES FOR BIOPRESERVATION OF BIOLOGICAL MATERIALS

CROSS REFERENCE TO RELATED APPLICATIONS

- 5 [0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/138,752, filed March 26, 2015, the contents of which are incorporated into the present application by reference.

BACKGROUND OF THE INVENTION

A. Field of the Invention

- 10 [0002] The invention generally relates to biopreservation of biological materials. In particular, the invention relates to the cryopreservation, thawing, and hypothermic preservation (cold storage) of cells, cell preparations, biological tissues and organs.

B. Description of Relevant Art

- 15 [0003] Biopreservation involves the preservation of biological materials including cells, cell preparations, biological tissues, and organs at cryogenic temperatures and/or hypothermic temperatures (cold storage). Generally, biological materials such as cells and cell preparations are preserved at cryogenic temperatures for long periods of time (e.g., up to 1 to 2 years), thawed, and then used immediately in uses such as tissue regeneration, wound healing, and cellular therapy.

- 20 [0004] For example, the cell preparation DERMAGRAFT® must be stored continuously at minus 65°C to minus 85°C in order to ensure cell viability, according to “DERMAGRAFT Directions for Use”, Organogenesis Inc., 2014, herein incorporated by reference. This requirement presents problems in shipping the cell preparation to the end user’s facility from the manufacturer, and in subsequent storage of the cell preparation at the
25 end user’s facility. Special shipping services must be employed and expensive cryogenic freezers or liquid nitrogen containers must be on site at the end user’s facility, such as hospitals, clinics, nursing facilities, or doctor’s offices, in order to maintain the cell preparation at cryogenic temperatures. Once thawed, DERMAGRAFT® must be immediately rinsed three times with saline solution to remove the hazardous cryoprotectant
30 solution containing DMSO from the cell preparation. After rinsing, the cell preparation must

be used within 30 minutes to ensure the delivery of living cells to the patient's wound; otherwise, the preparation should be discarded. These requirements are restrictive and problematic for the end user, because if the directions are not followed properly, the cells in the cell preparation will die and the preparation will not be efficacious.

- 5 [0005] Additional problems that are associated with the biopreservation of cells and cell preparations include the use of animal origin materials such as bovine serum and the use of hazardous materials such as dimethyl sulfoxide (DMSO). DMSO is commonly used as a cryoprotectant, but must be removed before the cells or cell preparation is used on humans because DMSO is irritating to the skin and mucous membranes. Bovine serum is also
10 commonly used in cryopreservation media because it can improve the viability and stability of the cells during the freezing process, storage at cryogenic temperatures, and subsequent thawing. However, the use of animal origin materials such as bovine serum are disadvantageous because they can introduce viruses and pathogenic contaminants into the biopreservation medium, plus the composition of the serum can vary from batch to batch.
- 15 [0006] Hypothermic (cold storage) media solutions such as HYPOTHERMOSOL® FRS available from BioLife Solutions, and PRIME-XV™ available from Irvine Scientific are commercially available for the transport and short term storage of biological materials; however, these solutions are not suitable for cryopreservation. Thus, if a manufacturer of a cell preparation were to thaw the cell preparation prior to shipping so that hypothermic
20 temperatures could be maintained during shipping and storage of the preparation at the end user's facilities, the manufacturer would have to remove the cryoprotectant medium from the cell preparation and add the hypothermic medium solution to the cell preparation before shipping. This process would present the opportunity for contamination of the preparation and potential loss of cell viability, plus this would entail additional handling costs at the
25 manufacturer.

SUMMARY OF THE INVENTION

- [0007] The present invention provides a solution to the aforementioned limitations and deficiencies in the art relating to biopreservation of biological materials. In one instance, the present invention provides for a biopreservation medium, and methods of use thereof,
30 such that the biopreservation medium can be used for each phase of biopreservation, i.e., cryopreservation, thawing, and hypothermic storage of biological materials such as cells, cell preparations, biological tissues and organs, carried out in succession without the need to

change the medium between phases. This eliminates the need to change the medium after thawing, and thus facilitates the shipment and short term storage of biological materials. The biological materials can remain under hypothermic conditions for at least 5 weeks which allows ample time for shipping and storing the biological materials at the end user's facility, such as a hospital, clinic, nursing facility, or doctor's office. It also eliminates the need for the end user to have a cryogenic freezer or liquid nitrogen on site. The end user only needs a refrigerator for storage of the thawed biological materials at refrigeration temperatures. Thus, in certain instances, the end user can even be a patient at home administering self-treatment, where the biological material could be stored in a home refrigerator.

[0008] Additionally, the biopreservation medium of the invention does not need the inclusion of DMSO for cryopreservation. The biopreservation medium of the invention has demonstrated a significant improvement of cell culture viability, quality and metabolic activity during cryopreservation and thawing over commercially available cryopreservation mediums such as SYNTH-A-FREEZE®, which contains 10% DMSO. Another advantage of the present invention is that only one medium is needed for all the phases of biopreservation, i.e. cryopreservation, thawing, and hypothermic storage, which is more economical than using a different medium for each phase. The biopreservation medium of the invention can also be animal origin free (AOF), since it does not need serum to promote cell viability during biopreservation and thawing.

[0009] In one aspect of the invention, there is disclosed a method for cryopreservation, thawing, and subsequent hypothermic storage of a cell preparation or a plurality of cells, the method comprising:

- a. combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture;
 - b. cryopreserving the mixture;
 - c. thawing the mixture; and
 - d. storing the thawed mixture at hypothermic conditions;
- wherein the viability of the cells is at least 50% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.

[0010] In one embodiment, the viability of the cells is at least 85% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.

- [0011] In one embodiment, the biopreservation medium composition is not changed between steps a and b. In further aspects, the biopreservation medium is not changed between steps c and d.
- [0012] In one embodiment, the one or more poloxamers includes poloxamer 407.
- 5 [0013] In one embodiment, the one or more poloxamers is poloxamer 407.
- [0014] In one embodiment, the one or more poloxamers does not include poloxamer 188.
- [0015] In one embodiment, the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.
- 10 [0016] In one embodiment, the one or more cryoprotectants includes glycerin.
- [0017] In one embodiment, the one or more cryoprotectants is glycerin.
- [0018] In one embodiment, the one or more cryoprotectants does not include DMSO.
- [0019] In one embodiment, the one or more cryoprotectants includes glycerin and does not include DMSO.
- 15 [0020] In one embodiment, the basal medium is animal origin free. In another embodiment, the biopreservation medium composition is animal origin free.
- [0021] In one embodiment, the plurality of cells comprises fibroblasts and/or keratinocytes. In another embodiment the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In
- 20 another embodiment, the plurality of cells comprises stem cells.
- [0022] In one embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes. In another embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the cell preparation comprises a carrier and stem cells.
- 25 [0023] In one embodiment, the viability of the cells is at least 60% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 weeks. In another embodiment, the viability of the cells is at least 90% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 weeks.
- [0024] In another aspect of the invention, there is disclosed a biopreservation medium
- 30 composition useful for the cryopreservation, thawing, and/or hypothermic preservation of a

cell preparation or a plurality of cells, the biopreservation medium comprising a basal medium, one or more poloxamers, and one or more cryoprotectants.

[0025] In one embodiment, the one or more poloxamers includes poloxamer 407.

[0026] In one embodiment, the one or more poloxamers is poloxamer 407.

5 [0027] In one embodiment, the one or more poloxamers does not include poloxamer 188.

[0028] In one embodiment, the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.

[0029] In one embodiment, the one or more cryoprotectants includes glycerin.

10 [0030] In one embodiment, the one or more cryoprotectants is glycerin.

[0031] In one embodiment, the one or more cryoprotectants does not include DMSO.

[0032] In one embodiment, the one or more cryoprotectants includes glycerin and does not include DMSO.

[0033] In one embodiment, the basal medium is animal origin free. In another
15 embodiment, the biopreservation medium composition is animal origin free.

[0034] In one embodiment, the plurality of cells comprises fibroblasts and/or keratinocytes. In another embodiment the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the plurality of cells comprises stem cells.

20 [0035] In one embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes. In another embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the cell preparation comprises a carrier and stem cells.

[0036] In one aspect of the invention, there is disclosed a method for the
25 cryopreservation of a cell preparation or a plurality of cells, the method comprising: combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture and cryopreserving the mixture. In one embodiment, the method further comprises thawing the cryopreserved mixture.

[0037] In one embodiment, the one or more poloxamers includes poloxamer 407.

[0038] In one embodiment, the one or more poloxamers is poloxamer 407.

[0039] In one embodiment, the one or more poloxamers does not include poloxamer 188.

5 [0040] In one embodiment, the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.

[0041] In one embodiment, the one or more cryoprotectants includes glycerin.

[0042] In one embodiment, the one or more cryoprotectants is glycerin.

[0043] In one embodiment, the one or more cryoprotectants does not include DMSO.

10 [0044] In one embodiment, the one or more cryoprotectants includes glycerin and does not include DMSO.

[0045] In one embodiment, the basal medium is animal origin free. In another embodiment, the biopreservation medium composition is animal origin free.

[0046] In one embodiment, the plurality of cells comprises fibroblasts and/or
15 keratinocytes. In another embodiment the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the plurality of cells comprises stem cells.

[0047] In one embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes. In another embodiment, the cell preparation comprises a carrier,
20 fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the cell preparation comprises a carrier and stem cells.

[0048] In another aspect of the invention, there is disclosed a method for the hypothermic storage of a cell preparation or a plurality of cells, the method comprising: combining the cell preparation or a plurality of cells with a biopreservation medium
25 composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture, and storing the thawed mixture at hypothermic conditions, wherein the viability of the cells is at least 50% of the viable cells initially present in the mixture after storage at hypothermic conditions for 5 weeks. In one embodiment, the viability of the cells is at least 85% of the viable cells initially present in the mixture after
30 storage at hypothermic conditions for 5 weeks.

[0049] In one embodiment, the viability of the cells is at least 60% of the viable cells initially present in the mixture after storage at hypothermic conditions for 2 weeks. In another embodiment, the viability of the cells is at least 90% of the viable cells initially present in the mixture after storage at hypothermic conditions for 2 weeks.

5 [0050] In one embodiment, the one or more poloxamers includes poloxamer 407.

[0051] In one embodiment, the one or more poloxamers is poloxamer 407.

[0052] In one embodiment, the one or more poloxamers does not include poloxamer 188.

[0053] In one embodiment, the one or more poloxamers includes poloxamer 407 and
10 does not include poloxamer 188.

[0054] In one embodiment, the one or more cryoprotectants includes glycerin.

[0055] In one embodiment, the one or more cryoprotectants is glycerin.

[0056] In one embodiment, the one or more cryoprotectants does not include DMSO.

[0057] In one embodiment, the one or more cryoprotectants includes glycerin and
15 does not include DMSO.

[0058] In one embodiment, the basal medium is animal origin free. In another embodiment, the biopreservation medium composition is animal origin free.

[0059] In one embodiment, the plurality of cells comprises fibroblasts and/or keratinocytes. In another embodiment the plurality of cells comprises fibroblasts and/or
20 keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the plurality of cells comprises stem cells.

[0060] In one embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes. In another embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically
25 inactivated. In another embodiment, the cell preparation comprises a carrier and stem cells.

[0061] The terms “cryogenic conditions”, “cryogenic temperatures”, or “cryopreservation temperatures” mean temperatures at minus 20°C to minus 196°C.

[0062] The terms “hypothermic conditions”, “hypothermic temperatures”, “refrigeration temperatures”, “refrigerated conditions” or “cold storage” mean temperatures at 0°C to 10 °C or 2°C to 8°C.

[0063] The term “animal origin free” (AOF) with respect to the biopreservation
5 mediums and basal mediums disclosed in the present invention means substantially free from animal derived components.

[0064] The term “about” or “approximately” are defined as being close to as understood by one of ordinary skill in the art, and in one non-limiting embodiment the terms are defined to be within 10%, preferably within 5%, more preferably within 1%, and most
10 preferably within 0.5%.

[0065] For this application, a number value with one or more decimal places can be rounded to the nearest whole number using standard rounding guidelines, i.e. round up if the number being rounded is 5, 6, 7, 8, or 9; and round down if the number being rounded is 0, 1, 2, 3, or 4. For example, 3.7 can be rounded to 4.

[0066] The words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0067] The use of the word “a” or “an” when used in conjunction with the terms “comprising”, “having”, “including”, or “containing” (or any variations of these words) may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[0068] The compositions and methods for their use can “comprise,” “consist
25 essentially of,” or “consist of” any of the ingredients or steps disclosed throughout the specification.

[0069] It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method or composition of the invention, and *vice versa*. Furthermore, compositions of the invention can be used to achieve methods of the invention.

[0070] Other objects, features and advantages of the present invention will become
30 apparent from the following detailed description. It should be understood, however, that the

detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

5

BRIEF DESCRIPTION OF THE DRAWINGS

[0071] **FIG. 1:** The cell viability of human keratinocytes during 5 week storage at 4°C in medium formulations from Examples 1, 2, and 3, and Hypothermosol.

[0072] **FIG. 2:** The number of cells (normalized) during 5 week storage at 4°C in medium formulations from Examples 1, 2, and 3, and Hypothermosol.

10

[0073] **FIG. 3:** Flow chart for experiment repeatability used in cryogenic study.

[0074] **FIG. 4:** Cell recovery results for medium formulas D, E, G, and H, and Synth-a-freeze used in cryogenic study, n=3.

[0075] **FIG. 5:** Cell recovery results for medium formulas D, E, G, and H, and Synth-a-freeze used in cryogenic study, n=2.

15

[0076] **FIG. 6:** Combined recovery results for medium formulas D, E, G, and H, and Synth-a-freeze used in cryogenic study, n=6.

[0077] **FIG. 7A:** Morphology micrographs (100X and 25X) at p4 of PIK41 Keratinocytes Post Thaw, Control Synth-a-freeze, formula D, and formula E.

[0078] **FIG. 7B:** Morphology micrographs (100X and 25X) at p4 of PIK41 Keratinocytes Post Thaw, formula G and formula H.

20

[0079] **FIG. 8:** Harvest results at p5 of the medium formulas D, E, G, and H, and Synth-a-freeze used in the cryogenic study.

[0080] **FIG. 9:** Thaw results of cryogenic study with recovery, viability and LDH values for PIK 41 keratinocytes in medium formulas D, E, G, and H, and Synth-a-freeze.

25

[0081] **FIG. 10:** Keratinocyte cell numbers in medium formulas D, E, G, and H, and Synth-a-freeze used throughout cryogenic study.

[0082] **FIG. 11:** The cell viability of human keratinocytes after 7 days storage at minus 80°C for medium formulas J, K, and L, and Synth-a-freeze.

[0083] **FIG. 12:** The number of viable cells after 7 days storage at minus 80°C for medium formulas J, K, and L, and Synth-a-freeze.

[0084] **FIG. 13:** The cell recovery results for medium formulas J, K, and L, and Synth-a freeze used in cryogenic study.

5 [0085] **FIG. 14:** LDH concentration for medium formulas J, K, and L, and Synth-a freeze used in cryogenic study.

[0086] **FIG. 15:** The cell viability of human keratinocytes after 7 days storage at minus 80°C for medium formulas M, N, and P, and Synth-a-freeze.

[0087] **FIG. 16:** The number of viable cells after 7 days storage at minus 80°C for
10 medium formulas M, N, and P, and Synth-a-freeze.

[0088] **FIG. 17:** The cell recovery results for medium formulas M, N, and P, and Synth-a freeze used in cryogenic study.

[0089] **FIG. 18:** LDH concentration for medium formulas M, N, and P, and Synth-a freeze used in cryogenic study.

15 [0090] **FIG. 19:** Viscosity results of medium formulas J, K, and L.

[0091] **FIG. 20:** Cell recovery as a function of viscosity of medium formulas J, K, and L, and Synth-a freeze.

DETAILED DESCRIPTION OF THE INVENTION

[0092] The present invention provides for a biopreservation medium composition and
20 methods of use thereof.

A. Biopreservation Medium Composition

[0093] The biopreservation medium of the present invention comprises a basal medium, one or more poloxamers, such as poloxamer 407, and one or more cryoprotectants, such as glycerin, and is suitable for the cryopreservation phase, thawing phase, and
25 hypothermic preservation phase (cold storage) of biological materials such as cells, cell preparations, biological tissues and organs. The advantage of the biopreservation medium of the present invention is that it can be used for all phases of biopreservation including cryopreservation, thawing, and hypothermic preservation carried out in succession without the need to change the medium between phases.

[0094] In one embodiment, there is disclosed a biopreservation medium composition useful for the cryopreservation, thawing, and hypothermic preservation of a cell preparation or a plurality of cells, the biopreservation medium comprising a basal medium, one or more poloxamers, and one or more cryoprotectants

5 [0095] The biopreservation medium of the present invention is also suitable for use in each phase of biopreservation separately and independent of the other phases. For example, the biopreservation medium of the present invention can be used solely as a cryopreservation medium, or solely as a thawing medium, or solely as a hypothermic (cold storage) medium. In one embodiment, the biopreservation medium is used solely as a cryopreservation
10 medium. In one embodiment, the biopreservation medium is used solely as a thawing medium. In one embodiment, the biopreservation medium is used solely as a hypothermic (cold storage) medium. In another embodiment, the biopreservation medium is used as the cryopreservation medium, the thawing medium, and the hypothermic (cold storage) medium.

[0096] The biopreservation medium of the present invention has also demonstrated
15 the ability to preserve cells at hypothermic conditions for up to 5 weeks while maintaining at least 50% cell viability. In one aspect of the invention, the viability of cells stored at hypothermic conditions for 5 weeks in the biopreservation medium of the present invention can be at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 85% of the cells initially present (in the thawed state). In another aspect
20 of the invention, the viability of cells stored at hypothermic conditions for 2 weeks in the biopreservation medium of the present invention can be at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% of the cells initially present (in the thawed state).

[0097] The biopreservation mediums of the present invention can be made by
25 blending the one or more poloxamers and the one or more cryoprotectants with a basal medium until a homogenous mixture is obtained using mixing and blending techniques and equipment known in the art. Non-limiting examples of suitable industrial mixing equipment include Lightnin' propeller-type mixers, Silverson rotor/stator homogenizers, and MorehouseCowles dissolvers. Use of aseptic techniques are desirable when producing the
30 mediums.

Animal Origin Free

[0098] The biopreservation medium of the present invention can be animal origin free (AOF), i.e., a medium substantially free from animal derived components. There are various disadvantages of using a medium containing serum and/or animal derived components, e.g. high cost, batch-to-batch variability of the serum and/or animal derived components, the risk of introducing viruses and pathogenic contaminants, and the difficulty in removing serum proteins and/or animal derived proteins from the biomaterials. An exemplary formulation of the present invention is AOF.

Basal Medium

[0099] Any basal medium compatible with the cells being preserved can be used in the present invention. The basal medium can be AOF. The basal medium can be obtained through commercial means from a manufacture or it can be manufactured in situ. EPILIFE® Basal Medium, commercially available from Life Technologies Corp., is suitable for use in the present invention. EPILIFE Basal Medium is especially compatible with human keratinocytes and human corneal epithelial cells. EPILIFE Basal Medium is AOF and is available with calcium chloride (60 μ M) or without calcium chloride. It is also available with or without phenol red. The components of EPILIFE Basal Medium are shown in Table 1 below.

Table 1

Amino Acids
Glycine
L-Alanine
L-Arginine hydrochloride
L-Asparagine-H ₂ O
L-Aspartic acid
L-Cysteine
L-Glutamic Acid
L-Glutamine
L-Histidine hydrochloride-H ₂ O

L-Isoleucine
L-Leucine
L-Lysine hydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine
L-Valine
Vitamins
Choline chloride
D-Pantothenic Acid
Folic Acid
Myo-Inositol
Niacinamide
Pyridoxal hydrochloride
Riboflavin
Thiamine hydrochloride
Vitamin B12
d-Biotin
Inorganic Salts
Ammonium Metavanadate NH_4VO_3
Ammonium Molybdate $((\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O})$
Calcium Chloride $(\text{CaCl}_2 \cdot 2\text{H}_2\text{O})$ (60 μM) (optional)
Cupric sulfate $(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})$
Ferric sulfate $(\text{FeSO}_4 \cdot 7\text{H}_2\text{O})$
Magnesium Chloride $(\text{MgCl}_2 \cdot 6\text{H}_2\text{O})$
Manganese Sulfate $(\text{MnSO}_4 \cdot \text{H}_2\text{O})$

Nickelous Chloride $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$
Potassium Chloride (KCl)
Sodium Bicarbonate (NaHCO_3)
Sodium Chloride (NaCl)
Sodium Meta Silicate $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$
Sodium Phosphate dibasic ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$)
Sodium Selenite (Na_2SeO_3)
Tin Chloride ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$)
Zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)
Other Components
Adenine.HCl
D-Glucose (Dextrose)
DL-alpha-Lipoic Acid
Ethanolamine
HEPES
O-Phosphorylethanolamine
Phenol Red (optional)
Putrescine 2HCl
Sodium Pyruvate
Thymidine

Poloxamers

[00100] The biopreservation medium of the present invention comprises one or more poloxamers, which are anhydrous, hydrophilic, nonionic, synthetic block copolymers of ethylene oxide and propylene oxide conforming to the general formula (I)



[00101] The term “poloxamer” is the nonproprietary name of the copolymer. Poloxamers are also known as PEO-PPO-PEO tri-block copolymers and can have various block lengths. Poloxamers are available in several types which have various physical forms and various average molecular weights. Each specific poloxamer type is identified by the

nonproprietary name “poloxamer” followed by a three digit number, the first two digits of which when multiplied by 100 correspond to the approximate average molecular weight of the polyoxypropylene portion of the copolymer; and the third digit, when multiplied by 10, corresponds to the percentage by weight of the polyoxyethylene portion.

5 [00102] Poloxamers are commercially available from BASF under the trade names PLURONIC®, LUTROL® and PLURACARE®; and poloxamers are also available from other suppliers. Poloxamers are available in solid and liquid forms. Solid forms are available as, but not limited to, flakes, prills, and pastes. Poloxamers are available in pharmaceutical, cosmetic, and industrial grades. Pharmaceutical grade poloxamers are listed in recognized
10 pharmaceutical compendia such as USP/NF and European Pharmacopeia (PhEur). According to the USP/NF and PhEur, a suitable antioxidant may be added.

[00103] Pharmaceutical grades of the poloxamers listed in the USP/NF are shown in Table 2 below. Also shown are the physical form, average molecular weight, and *a* and *b* values corresponding to formula (I) for each poloxamer.

15 **Table 2 – USP/NF Grades of Poloxamers**

Poloxamer	Physical form	<i>a</i>	<i>b</i>	Average molecular weight
124	Liquid	12	20	2090-2360
188	Solid	80	27	7680-9510
237	Solid	64	37	6840-8830
338	Solid	141	44	12700-17400
407	Solid	101	56	9840-14600

[00104] Poloxamer 407, also known by its trade name PLURONIC F127, is suitable for use in the present invention. The data reveal that Poloxamer 188, also known by its trade name PLURONIC F68, was not as effective as Poloxamer 407 in maintaining cell viability after storage of cells at hypothermic conditions in the biopreservation medium formulation of
20 the present invention.

[00105] In various embodiments, the concentration of poloxamers in the biopreservation medium of the present invention can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25% w/w or any percentage derivable therein of the total composition weight. In other embodiments, the concentration of poloxamers can be

2% w/w, or 5% w/w, or 6 % w/w, or 7% w/w, or 8% w/w, or 10% w/w, or 11% w/w, or 12 % w/w, or 20 % w/w of the total composition weight. In other embodiments, the concentration of poloxamers can be 2-20 % w/w, or 2-12% w/w, or 5-20 % w/w, or 5-12% w/w, or 5-11% w/w, or 5-10% w/w, or 6-20% w/w, or 6-12% w/w, or 6-11%w/w, or 6-10% w/w of the total composition weight.

Cryoprotectants

[00106] Various cryoprotectants known in the art may be used in the present invention as long as they are not toxic to human tissue. For example, DMSO is widely used as a cryoprotectant of cells, but it is toxic (irritating) to the skin and mucous membranes; thus, DMSO is not a suitable cryoprotectant for use in the present invention. Glycerin, also known as glycerol, is a suitable cryoprotectant for use in the present invention. For use in an AOF biopreservation medium, a non-animal source of glycerin is preferred, such as a synthetic or vegetable source of glycerin both of which are widely available commercially. In various embodiments, the concentration of the cryoprotectant in the biopreservation medium of the present invention can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25% w/w or any percentage derivable therein of the total composition weight. In other embodiments, the concentration of the cryoprotectant can be 4% w/w, or 8% w/w, or 16% w/w of the total composition weight. In other embodiments, the concentration of the cryoprotectant can be 4-16% w/w, or 4-8% w/w, or 8-16% w/w.

B. Cells and Cell Preparations

Cell Types

[00107] The biopreservation medium of the present invention is suitable for the biopreservation of a variety of cell types including animal (mammalian and non-mammalian) and plant cells. The biopreservation medium of the present invention is suitable for the biopreservation of one cell, a plurality of cells which can be of one cell type or of mixed cell types, a population of cells of one cell type, or a population of mixed cells.

[00108] Non-limiting examples of mammalian animal cell types include fibroblasts, keratinocytes, dermal cells, melanocytes, hair cells, outer root sheath cells, epithelial cells, corneal epithelial cells, progenitor cells, stromal cells, lymphocytes including plasma cells, B cells, T cells, cytotoxic T cells, natural killer T cells, regulatory T cells, T helper cells, myeloid cells, granulocytes, basophil granulocytes, eosinophil granulocytes, neutrophil

granulocytes, hypersegmented neutrophils, monocytes, macrophages, reticulocytes, platelets, mast cells, thrombocytes, megakaryocytes, dendritic cells, thyroid cells, thyroid epithelial cells, parafollicular cells, parathyroid cells, parathyroid chief cells, oxyphil cells, adrenal cells, chromaffin cells, pineal cells, pinealocytes, glial cells, glioblasts, astrocytes, oligodendrocytes, microglial cells, magnocellular neurosecretory cells, stellate cells, boettcher cells; pituitary cells, gonadotropes, corticotropes, thyrotropes, somatotrope, lactotrophs, pneumocyte, type I pneumocytes, type II pneumocytes, Clara cells; goblet cells, alveolar macrophages, myocardiocytes, pericytes, gastric cells, gastric chief cells, parietal cells, goblet cells, paneth cells, G cells, D cells, ECL cells, I cells, K cells, S cells, enteroendocrine cells, enterochromaffin cells, APUD cell, liver cells, hepatocytes, Kupffer cells, bone cells, osteoblasts, osteocytes, osteoclast, odontoblasts, cementoblasts, ameloblasts, cartilage cells, chondroblasts, chondrocytes, trichocytes, nevus cells, muscle cells, neurons, nerve cells, neuroglia cells, myocytes, myoblasts, myotubes, adipocytes, tendon cells, podocytes, juxtaglomerular cells, intraglomerular mesangial cells, extraglomerular mesangial cells, kidney cells, macula densa cells, spermatozoa, sertoli cells, leydig cells, oocytes, caco-2 cells, blood-derived stem cells, umbilical cord blood cells, mononuclear cells, differentiated cells, differentiated fibroblasts, mitotically active, mitotically inactive cells, irradiated cells, autologous cells, allogeneic cells, xenogeneic, isograft cells, allograft cells, xenograft cells, genetically engineered cells, immortalized cells, and stem cells including mammalian and non-mammalian stem cells.

[00109] Non limiting examples of stem cells include adult stem cells, fetal stem cells, embryonic stem cells, pluripotent stem cells, induced pluripotent stem cells, multipotent stem cells, totipotent stem cells, oligopotent stem cells, unipotent stem cells, mesenchymal stem cells, amniotic stem cells, umbilical cord blood stem cells, bone marrow stem cells, hematopoietic stem cells, endothelial stem cells, adipose stem cells, dental pulp stem cells, cancer stem cells.

[00110] In one embodiment, the biological material is a plurality of cells. In another embodiment, the plurality of cells comprises fibroblasts and/or keratinocytes. In another embodiment the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the plurality of cells comprises stem cells.

Cell Preparations

[00111] The biopreservation medium of the present invention is suitable for the biopreservation of cell preparations. Cell preparations are known in the art and are useful for a variety of medical and clinical uses including, but not limited to, tissue regeneration, wound healing, and cellular therapy. Cell preparations include, but are not limited to, cells in or on a carrier, substrate or matrix; a suspension of cells in a culture medium or carrier; skin equivalents; and skin substitutes. An example of a cell preparation is DERMAGRAFT®, available from Organogenesis, Inc. Other examples of cell preparations are the cell preparations disclosed in US patents US 6673603, US 7144729, US 7449333, US 7700351, US 7879605, US 8137965, US 8323638, and US 8679475 all of which are herein incorporated by reference.

[00112] In one embodiment, the biological material is a cell preparation. In another embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes. In another embodiment, the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated. In another embodiment, the cell preparation comprises a carrier and stem cells.

C. Biological Tissues and Organs

[00113] The biopreservation medium of the present invention is suitable for the biopreservation of a variety of biological tissues and organs including animal (mammalian and non-mammalian) tissues and organs. Non-limiting examples of mammalian animal tissues include chondral tissue, cartilage, tendons, ligaments, vertebral discs, osteochondral tissue, islet tissue, osteogenic tissue, neural tissue, skin, mucous membranes, bone tissue, bone marrow, adipose tissue, muscle tissue, blood, corneas, lens, ocular tissue, meniscus, hair follicles, striated muscle, smooth muscle, cardiac muscle, connective tissue, blood vessels, functional spine units, muscle-tendon grafts. Non-limiting examples of organs include heart, liver, and kidney.

D. Biopreservation Methods

[00114] In one aspect of the invention the biopreservation method of the invention relates to the cryopreservation, thawing, and hypothermic preservation (cold storage) of cells, cell preparations, biological tissues and organs, carried out in succession using the biopreservation medium composition of the invention without changing the biopreservation

medium composition at any time throughout the biopreservation method. In another aspect of the invention, the biopreservation method of the invention relates to the cryopreservation of cells, cell preparations, biological tissues and organs using the biopreservation medium composition of the invention. In another aspect of the invention, the biopreservation method of the invention relates to the thawing of cells, cell preparations, biological tissues and organs from cryogenic conditions using the biopreservation medium composition of the invention. In another aspect of the invention, the biopreservation method of the invention relates to the hypothermic preservation (cold storage) of cells, cell preparations, biological tissues and organs using the biopreservation medium composition of the invention. It was demonstrated that the biopreservation medium of the present invention retained a significantly higher cell viability percentage after 5 weeks of hypothermic storage than the commercially available hypothermic storage medium HYPOTHERMASOL®.

[00115] In one embodiment there is disclosed a method for cryopreservation, thawing, and subsequent hypothermic storage of a cell preparation or a plurality of cells, the method comprising:

- a. combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture;
 - b. cryopreserving the mixture;
 - c. thawing the mixture; and
 - d. storing the thawed mixture at hypothermic conditions;
- wherein the viability of the cells is at least 50% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.

[00116] In one embodiment, the viability of the cells is at least 85% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.

[00117] In one embodiment the viability of the cells is at least 60% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 weeks. In another embodiment, the viability of the cells is at least 90% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 week.

Cryopreservation

[00118] Cryopreservation techniques for cryopreserving cells, cell preparations, biological tissues and organs are known in the art. Generally, the cryopreservation temperatures are between minus 20°C to minus 196°C. Cryogenic freezers are commonly
5 used for cryopreservation and are commercially available. The use of liquid nitrogen in storage tanks is another known technique for cryopreservation.

Thawing

[00119] Techniques for thawing biological materials from cryogenic conditions are known in the art. Care must be exercised during thawing to prevent damage or death to cells
10 and/or tissues. Generally, water baths or electric heating devices are used for thawing cryopreserved biological materials. For example, the devices and methods for thawing biological materials disclosed in International Patent Application, publication number WO 2013/126379, herein incorporated by reference, are suitable for the present invention.

Hypothermic storage (Cold Storage)

[00120] Techniques for hypothermic storage (cold storage) of biological materials are known in the art and include the transport and storage of the materials. Hypothermic conditions include temperatures at 0°C to 10 °C or 2°C to 8°C. Ordinary household or commercial grade refrigerators can be used for hypothermic storage. Care must be exercised
15 during the transport of biological materials to prevent exposure of the materials to temperatures above hypothermic conditions. Biological materials can be shipped via refrigerated trucks or rail cars. Insulated shipping containers with cold packs can be used for shipment by mail or express delivery services. Ice chests with cold packs or ice can also be
20 employed for transport in automobiles.

EXAMPLES

[00121] The following examples are included to demonstrate certain non-limiting aspects of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the applicants to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1
(Biopreservation Medium – Poloxamer 407/Glycerin Based)

Table 3

Ingredient	% w/w
Poloxamer 407	6
Glycerin	4.3
EPILIFE® Basal Medium w/ 60 μ M calcium	qs ad 100%

[00122] Poloxamer 407 was added to the basal medium and mixed at 4°C until dissolved. Glycerin was added and blended until homogenous at 4°C. The solution was filtered through a 0.2 μ m filter into a sterile container.

Example 2
(Biopreservation Medium – Poloxamer 407/Glycerin Based)

Table 4

Ingredient	% w/w
Poloxamer 407	11
Glycerin	4.3
EPILIFE® Basal Medium w/ 60 μ M calcium	qs ad 100%

[00123] Poloxamer 407 was added to the basal medium and mixed at 4°C until dissolved. Glycerin was added and blended until homogenous at 4°C. The solution was filtered through a 0.2 μ m filter into a sterile container.

Example 3
(HEC Based Medium)

Table 5

Ingredient	% w/w
Hydroxyethylcellulose (HEC)	0.14
Glycerin	8.3
EPILIFE® Basal Medium w/ 60 µM calcium	qs ad 100%

5 [00124] The ingredients were blended until homogenous.

Example 4
(Cell Viability in Biopreservation Medium at Hypothermic Temperatures)

[00125] Human keratinocytes from the fifth passage of cell line PIK 48 were seeded into T75 flasks and collected after 5 days. The collected cells were then suspended into 30 mL of 2H media, which is a medium comprising EpiLife® Basal Medium + HKSdaFree™ + HKGE™. HKSdaFree and HKGE are available from AvantBio Corp, Vancouver, Washington. Cell counts were obtained (1.0×10^6 total cells per mL) and 2 mL aliquots were placed into 15 mL tubes. Cells were spun down in the centrifuge and the media was aspirated away. The cells were added to 1 mL of each biopreservation medium of Example 1, Example 2, and Example 3 and transferred to 2 mL Nunc vials. Human keratinocytes were also added to Hypothermasol® as a control. All samples were stored at 2-8°C for 35 days (five weeks). The cell viability and cell count of each sample was determined periodically using a NucleoCounter® NC-3000 cell analyzer. The results are shown in Fig. 1 and Fig. 2. As can be seen in the results, the Biopreservation mediums of Example 1 and Example 2 showed a cell viability of greater than 90% at 15 days (two weeks) and greater than 85% at 35 days (five weeks). Example 3 showed a cell viability of 20% at 15 days. Hypothermasol showed a cell viability of greater than 85% at 15 days, but showed a cell viability of less than 60% at 35 days.

Example 5
(Various Medium Formulations and Hypothermic Cell Viability Study)

[00126] The following medium formulations in Table 6 below were prepared and were used in a cell viability study at hypothermic conditions.

Table 6

Formula	Poloxamer 407 % w/w	Glycerin % w/w	Poloxamer 188 % w/w	Dextran 40 % w/w	EpiLife® w/60 µM calcium
AA	-	-	-	2	qs ad 100%
BB	-	-	-	5	qs ad 100%
CC	-	-	-	10	qs ad 100%
DD	6	4.3	-	-	qs ad 100%
EE	10	4.3	-	-	qs ad 100%
FF	6	-	-	-	qs ad 100%
GG	10	-	-	-	qs ad 100%
HH	-	4.3	10	-	qs ad 100%
II	-	4.3	20	-	qs ad 100%
JJ	-	-	10	-	qs ad 100%
KK	-	-	20	-	qs ad 100%

[00127] Human keratinocytes from the fifth passage of cell line PIK 48 were seeded into T75 flasks and collected after 5 days. The collected cells were then suspended into 30 mL of 2H media. Cell counts were obtained (1.0×10^6 total cells per mL) and 2 mL aliquots were placed into 15 mL tubes. Cells were spun down in the centrifuge and the media was aspirated away. The cells were added to 1 mL of each medium in Table 3 and transferred to 2 mL Nunc vials. Human keratinocytes were also added to Hypothermasol®, EpiLife® Basal medium w/60 µM calcium, and 2H Media. All samples were stored at 2-8°C for 14 days (two weeks). The cell viability and cell count of each sample was determined at 7 and 14 days using a NucleoCounter® NC-3000 cell analyzer. Samples were run twice and the average values were used in the results shown in Table 7 below.

Table 7

	% Cell Viability		Total Cells $\times 10^6$ 14 day	Viable Cells $\times 10^6$ 14 day
	7 day	14 day		
HypoThermosol®	93.9	92.9	1.98	1.84
EpiLife® w/60 µM calcium	9.6	nr	nr	-
Formula AA	6.3	4.3	1.53	0.06
Formula BB	19.6	5.8	0.92	0.05
Formula CC	63.9	25.0	0.89	0.22
Formula DD	92.8	91.5	1.13	1.03
Formula EE	93.1	92.7	0.92	0.85
Formula FF	90.8	86.1	1.07	0.92

Formula GG	89.7	89.7	1.10	0.99
Formula HH	76.3	63.6	0.90	0.58
Formula II	83.6	80.0	0.80	0.64
Formula JJ	74.7	51.1	0.90	0.46
Formula KK	nr	89.0	1.23	1.09
2H media	22.5	nr	nr	-

[00128] As can be seen in the results shown in Table 7, medium formulations containing Poloxamer 407 and glycerin in EpiLife® Basal medium w/60 μ M calcium, i.e. formulas DD and EE, showed a cell viability of greater than 90% at 14 days (two weeks).
5 HypoThermosol® also showed a cell viability of greater than 90% at 15 days (two weeks). Medium formulations containing Poloxamer 188 and glycerin, i.e. formulas HH and II, showed a cell viability of less than 90% at 14 days (two weeks).

Example 6 (Various Mediums and Cryogenic Cell Viability Study I)

10 [00129] The following medium formulations in Table 8 below were prepared and used in a cell viability study at cryogenic conditions. Synth-a-freeze® cryopreservation medium was also used in the study as a control.

Table 8

Formula	Poloxamer 407 % w/w	Glycerin % w/w	Hydroxyethylcellulose %w/w	EpiLife® w/60 μ M calcium
D	-	8.29	0.14	qs ad 100%
E	-	16.71	0.13	qs ad 100%
G	5.55	16.77	-	qs ad 100%
H	5.12	7.7	-	qs ad 100%

15 [00130] Human keratinocytes (PIK 41) were cryopreserved in vials at minus 80°C in each medium listed in Table 8. The cells were also cryopreserved in Synth-a-freeze® (SAF) cryopreservation medium as a control. Triplicate and duplicate vials were created and thawed for repeatability as outlined in Fig. 3. After 10 days, the vials were thawed using a water bath to 37 $\pm$ 0.5°C temperature. The cell viability and cell count of each sample was
20 determined using a NucleoCounter® NC-3000 cell analyzer. The cell viability and recovery

results are shown in Fig. 4, Fig. 5, and Fig 6. Formulations D and H consistently performed better than the control Synth-a-freeze. Formulation E consistently had the lowest viability.

[00131] All formulations were plated on T75 flasks in culture and placed in an incubator at 37°C in 5% carbon dioxide, 95% air. Typically seeding density is dependent on the number of viable cells recovered, but to better compare the different media, the cultures were seeded with a fixed amount: 0.26mL of an expected 1×10^6 cells/mL cell suspension. Pictures were taken and cells were harvested when the flasks were confluent, four days later. Fig. 7A and Fig. 7B depict morphology and confluence of the four cryopreservation formulations compared to the control.

[00132] Harvest results shown in Fig 8 show that formulations D and H performed better than the control. Formulation H yielded more cells, while formulation D produced more population doublings. Population doubling calculates how many times the population doubles given the initial seeding and final harvest.

[00133] Fig. 9 depicts each result obtained from the thaw performed from cells harvested at p5. The supernatant from the cells was measured on the Cedex Bio Analyzer to determine LDH (lactate dehydrogenase) levels in the freezing media. LDH has been shown to reflect the amount of dead cells in a cell suspension. Fig. 9 confirms that higher LDH reflects more dead cells, resulting in lower recovery. Both Synth-a-Freeze® and formulation E had undesirably high LDH levels, reflecting the lower recovery.

[00134] Fig. 10 depicts the cells at each stage of the experiment. The cells were originally frozen at the same density (triplicate vials), recovered and plated at passage 4 (triplicate flasks), harvested and frozen at passage 5 (duplicate vials), and finally recovered from thaw. Due to the greater cell harvest and recovery achieved by formulation H, the number of cells at the end of the experiment increased by 30%. Formulation D still did relatively well, with only a loss of 5%. The control Synth-a-Freeze lost the most cells overall, with a drop of 26%.

Example 7
(Various Mediums and Cryogenic Cell Viability Study II)

[00135] The following medium formulations in Table 9 below were prepared and used in a cell viability study at cryogenic conditions.

5

Table 9

Formula	Poloxamer 407 % w/w	Glycerin % w/w	EpiLife® w/60 μM calcium
J	2	3.7	qs ad 100%
K	7.7	3.7	qs ad 100%
L	12	3.7	qs ad 100%
M	7	-	qs ad 100%
N	7	3.7	qs ad 100%
P	7	11	qs ad 100%

[00136] Human keratinocytes were cryopreserved in vials at minus 80°C in each medium listed in Table 9. The cells were also cryopreserved in Synth-a-freeze® (SAF) cryopreservation medium as a control. After 7 days, the vials were thawed using a water bath to 37±0.5°C temperature. The cell viability and cell count of each sample was determined using a NucleoCounter® NC-3000 cell analyzer. The supernatant from the cells was measured on the Cedex Bio Analyzer to determine LDH (lactate dehydrogenase) levels in the freezing media. The cell viability, count, recovery, LDH concentration results are shown in Figs. 11 – 18. As can be seen in the results, the % cell recovery of formulas K, L, N and P was higher than the Synth-a-freeze control. Also, the LDH concentration was lower for formulas K, L, N and P than the Synth-a-freeze control indicating healthier cells.

10

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Example 8
(Viscosity of Mediums)

[00137] The viscosity of formulas J, K, and L, of Example 7 was taken using a Brookfield RV DV-E viscometer using Spindle #3 at 50 rpm for 1 min. at room temperature (RT) and the results are shown in Fig. 19. Viscosity was mostly dependent on poloxamer 407 concentrations in the mediums. Cell recovery as a function of viscosity was plotted and is shown in Fig 20.

20

CLAIMS

1. A method for cryopreservation, thawing, and subsequent hypothermic storage of a cell preparation or a plurality of cells, the method comprising:
 - a. combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture;
 - b. cryopreserving the mixture;
 - c. thawing the mixture; and
 - d. storing the thawed mixture at hypothermic conditions, wherein the viability of the cells is at least 50% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.
2. The method of claim 1, wherein the biopreservation medium composition is not changed between steps a and b, and between steps c and d.
3. The method of claim 1, wherein the one or more poloxamers includes poloxamer 407.
4. The method of claim 1, wherein the one or more poloxamers is poloxamer 407.
5. The method of claim 1, wherein the one or more poloxamers does not include poloxamer 188.
6. The method of claim 1, wherein the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.
7. The method of claim 1, wherein the one or more cryoprotectants includes glycerin.
8. The method of claim 1, wherein the one or more cryoprotectants is glycerin.
9. The method of claim 1, wherein the one or more cryoprotectants does not include DMSO.
10. The method of claim 1, wherein the one or more cryoprotectants includes glycerin and does not include DMSO.
11. The method of claim 1, wherein the basal medium is animal origin free.
12. The method of claim 1, wherein the biopreservation medium composition is animal origin free.

13. The method of claim 1, wherein the plurality of cells comprises fibroblasts and/or keratinocytes.
14. The method of claim 1, wherein the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
15. The method of claim 1, wherein the plurality of cells comprises stem cells.
16. The method of claim 1, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes.
17. The method of claim 1, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
18. The method of claim 1, wherein the viability of the cells is at least 85% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 5 weeks.
19. The method of claim 1, wherein the viability of the cells is at least 60% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 weeks.
20. The method of claim 1, wherein the viability of the cells is at least 90% of the viable cells initially present in the thawed mixture after storage at hypothermic conditions for 2 weeks.
21. A biopreservation medium composition useful for the cryopreservation, thawing, and/or hypothermic preservation of a cell preparation or a plurality of cells, the biopreservation medium comprising a basal medium, one or more poloxamers, and one or more cryoprotectants.
22. The composition of claim 21, wherein the one or more poloxamers includes poloxamer 407.
23. The composition of claim 21, wherein the one or more poloxamers is poloxamer 407.
24. The composition of claim 21, wherein the one or more poloxamers does not include poloxamer 188.

25. The composition of claim 21, wherein the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.
26. The composition of claim 21, wherein the one or more cryoprotectants includes glycerin.
27. The composition of claim 21, wherein the one or more cryoprotectants is glycerin.
28. The composition of claim 21, wherein the one or more cryoprotectants does not include DMSO.
29. The composition of claim 21, wherein the one or more cryoprotectants includes glycerin and does not include DMSO.
30. The composition of claim 21, wherein the basal medium is animal origin free.
31. The composition of claim 21, wherein the biopreservation medium composition is animal origin free.
32. The composition of claim 21, wherein the plurality of cells comprises fibroblasts and/or keratinocytes.
33. The composition of claim 21, wherein the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
34. The composition of claim 21, wherein the plurality of cells comprises stem cells.
35. The composition of claim 21, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes.
36. The composition of claim 21, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
37. The composition of claim 21, wherein the cell preparation comprises a carrier and stem cells.
38. A method for the cryopreservation of a cell preparation or a plurality of cells, the method comprising: combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture and cryopreserving the mixture.

39. The method of claim 38, further comprising thawing the cryopreserved mixture.
40. The method of claim 38 or 39, wherein the one or more poloxamers includes poloxamer 407.
41. The method of claim 38 or 39, wherein the one or more poloxamers is poloxamer 407.
42. The method of claim 38 or 39, wherein the one or more poloxamers does not include poloxamer 188.
43. The method of claim 38 or 39, wherein the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.
44. The method of claim 38 or 39, wherein the one or more cryoprotectants includes glycerin.
45. The method of claim 38 or 39, wherein the one or more cryoprotectants is glycerin.
46. The method of claim 38 or 39, wherein the one or more cryoprotectants does not include DMSO.
47. The method of claim 38 or 39, wherein the one or more cryoprotectants includes glycerin and does not include DMSO.
48. The method of claim 38 or 39, wherein the basal medium is animal origin free.
49. The method of claim 38 or 39, wherein the biopreservation medium composition is animal origin free.
50. The method of claim 38 or 39, wherein the plurality of cells comprises fibroblasts and/or keratinocytes.
51. The method of claim 38 or 39, wherein the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
52. The method of claim 38 or 39, wherein the plurality of cells comprises stem cells.
53. The method of claim 38 or 39, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes.

54. The method of claim 38 or 39, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
55. The method of claim 38 or 39, wherein the cell preparation comprises a carrier and stem cells.
56. A method for the hypothermic storage of a cell preparation or a plurality of cells, the method comprising: combining the cell preparation or a plurality of cells with a biopreservation medium composition comprising a basal medium, one or more poloxamers, and one or more cryoprotectants, to form a mixture, and storing the thawed mixture at hypothermic conditions, wherein the viability of the cells is at least 50% of the viable cells initially present in the mixture after storage at hypothermic conditions for 5 weeks.
57. The method of claim 56, wherein the viability of the cells is at least 60% of the viable cells initially present in the mixture after storage at hypothermic conditions for 2 weeks.
58. The method of claim 56, wherein the one or more poloxamers includes poloxamer 407.
59. The method of claim 56, wherein the one or more poloxamers is poloxamer 407.
60. The method of claim 56, wherein the one or more poloxamers does not include poloxamer 188.
61. The method of claim 56, wherein the one or more poloxamers includes poloxamer 407 and does not include poloxamer 188.
62. The method of claim 56, wherein the one or more cryoprotectants includes glycerin.
63. The method of claim 56, wherein the one or more cryoprotectants is glycerin.
64. The method of claim 56, wherein the one or more cryoprotectants does not include DMSO.
65. The method of claim 56, wherein the one or more cryoprotectants includes glycerin and does not include DMSO.

66. The method of claim 56, wherein the basal medium is animal origin free.
67. The method of claim 56, wherein the biopreservation medium composition is animal origin free.
68. The method of claim 56, wherein the plurality of cells comprises fibroblasts and/or keratinocytes.
69. The method of claim 56, wherein the plurality of cells comprises fibroblasts and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
70. The method of claim 56, wherein the plurality of cells comprises stem cells.
71. The method of claim 56, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes.
72. The method of claim 56, wherein the cell preparation comprises a carrier, fibroblasts, and/or keratinocytes, wherein the fibroblasts and/or keratinocytes are mitotically inactivated.
73. The method of claim 56, wherein the cell preparation comprises a carrier and stem cells.

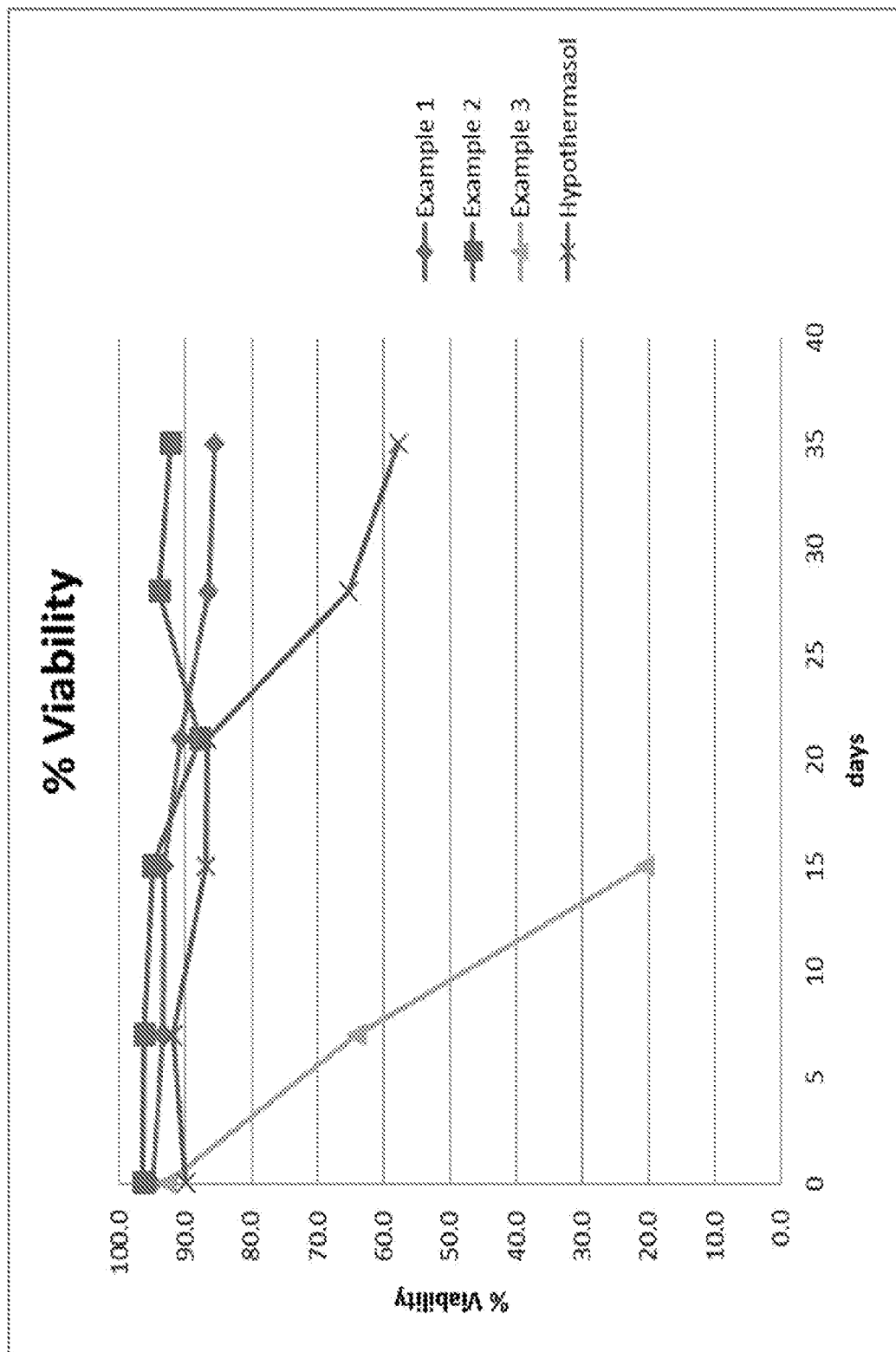


FIG. 1

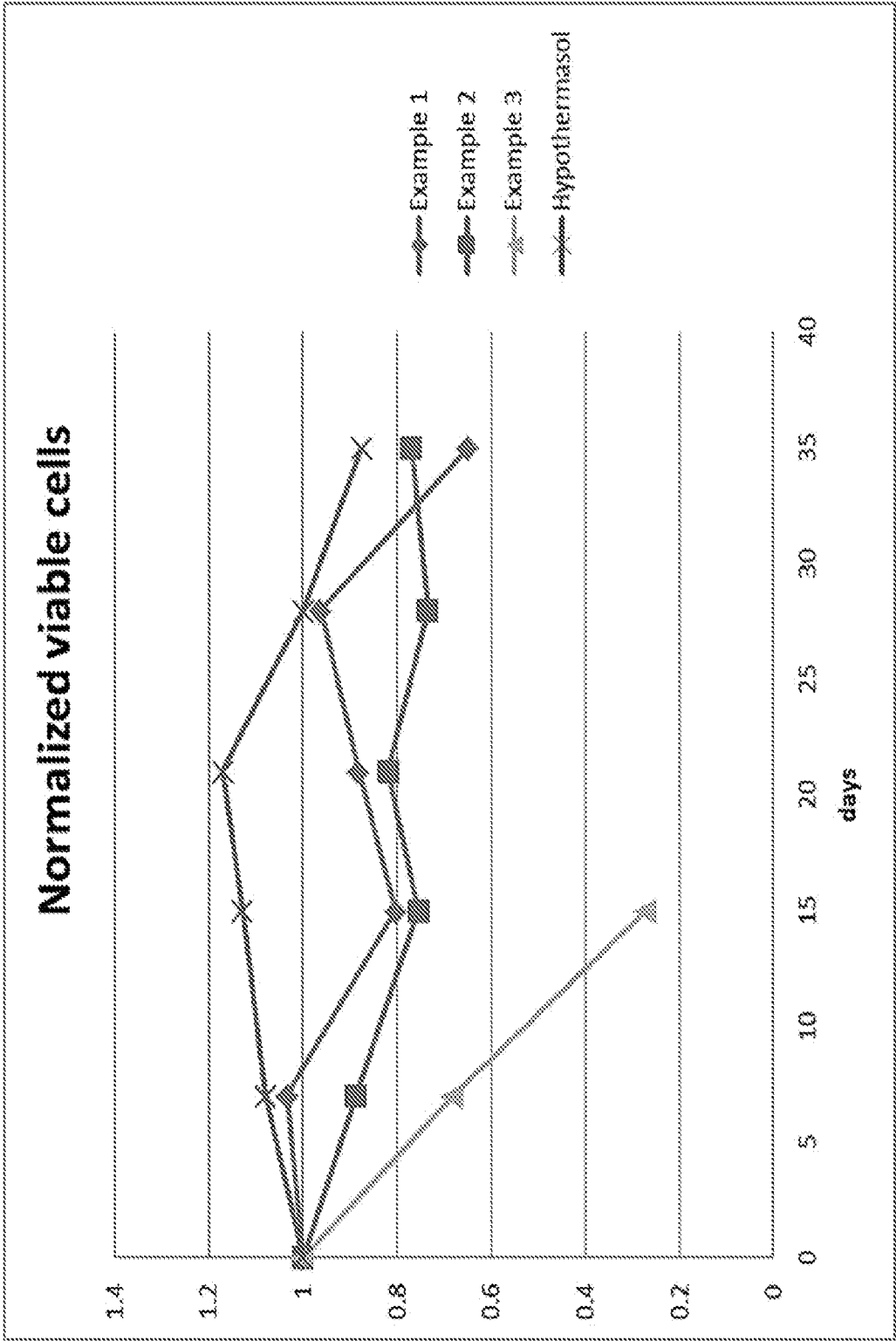
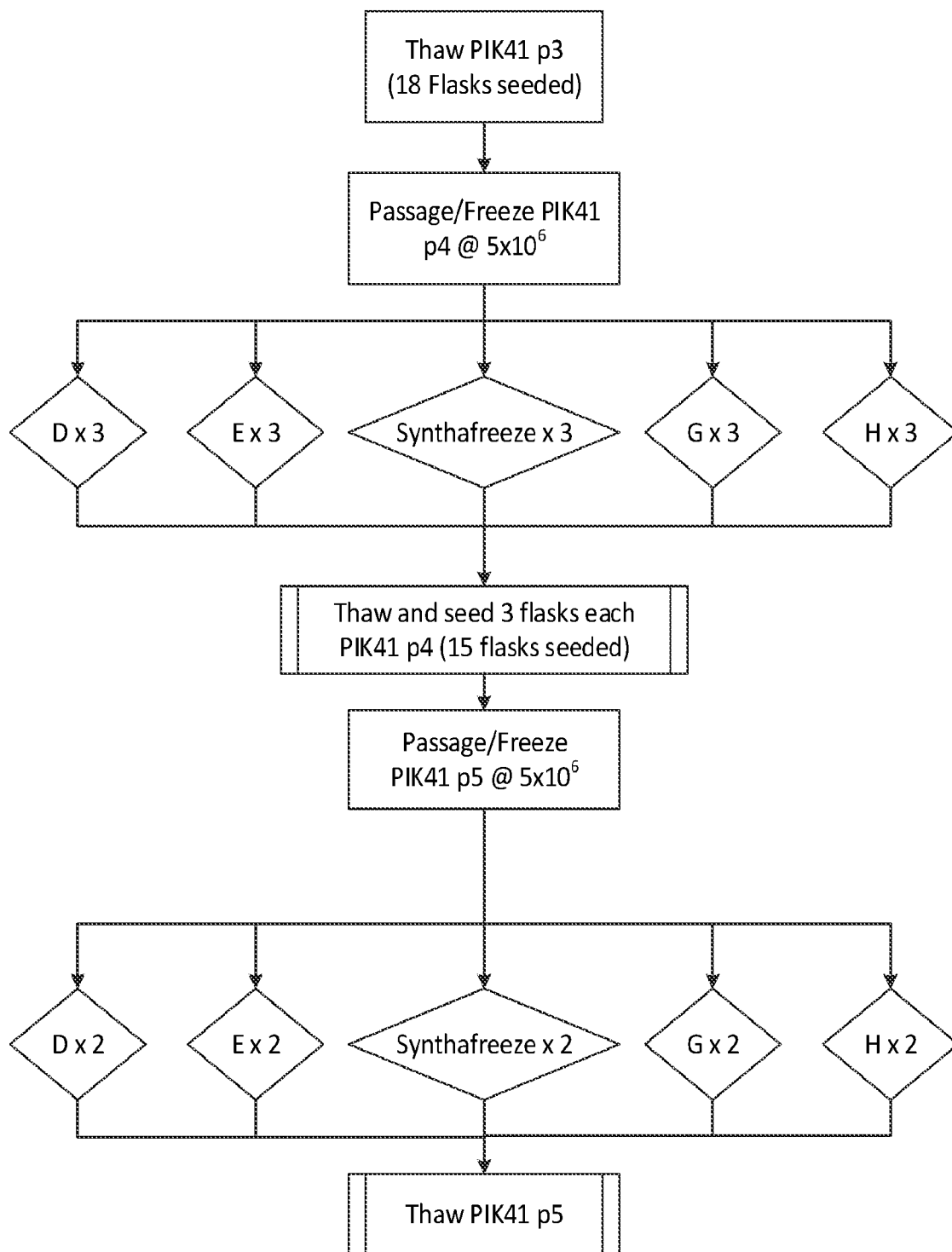


FIG. 2

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Flow Chart for Experiment Repeatability**FIG. 3**

Recovery Results for 4 Cryopreservation Medium Formulations, n=3

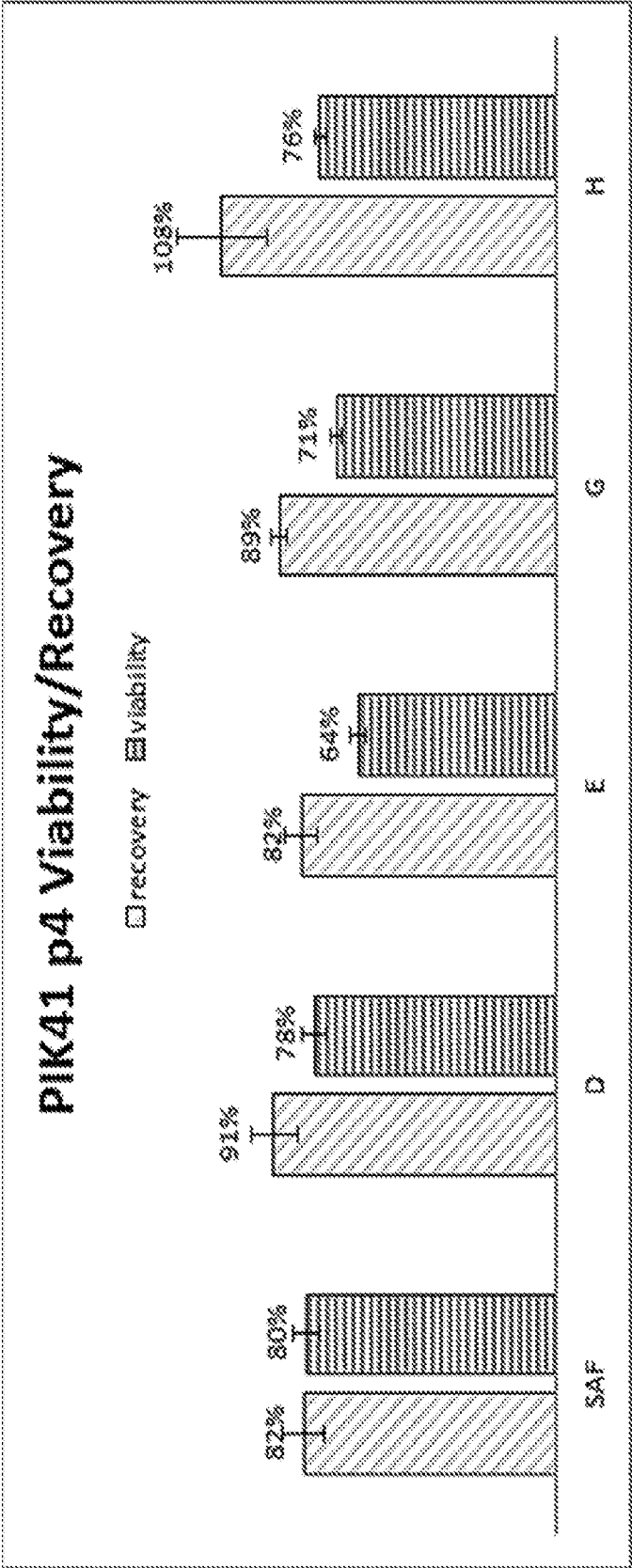


FIG. 4

Recovery Results for 4 Cryopreservation Medium Formulations, n=2

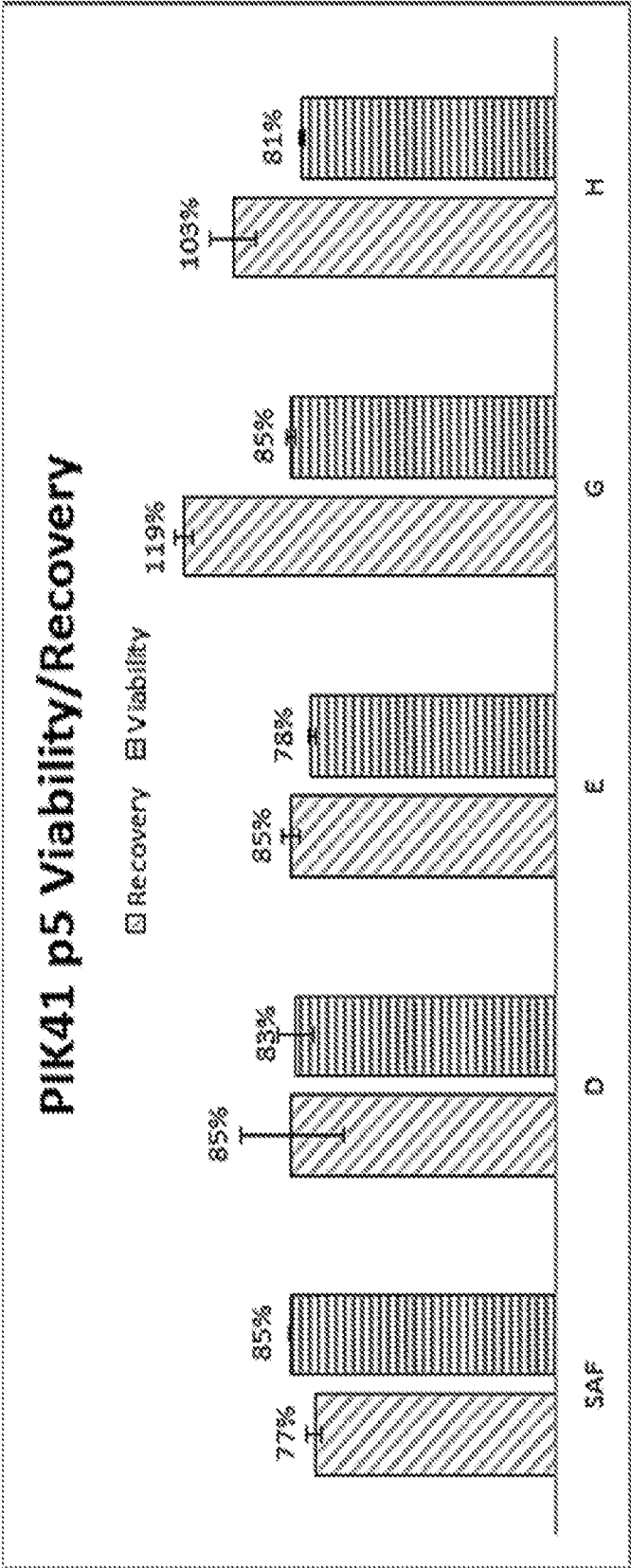


FIG. 5

Combined Recovery Results for 4 Cryopreservation Medium Formulations, n=6

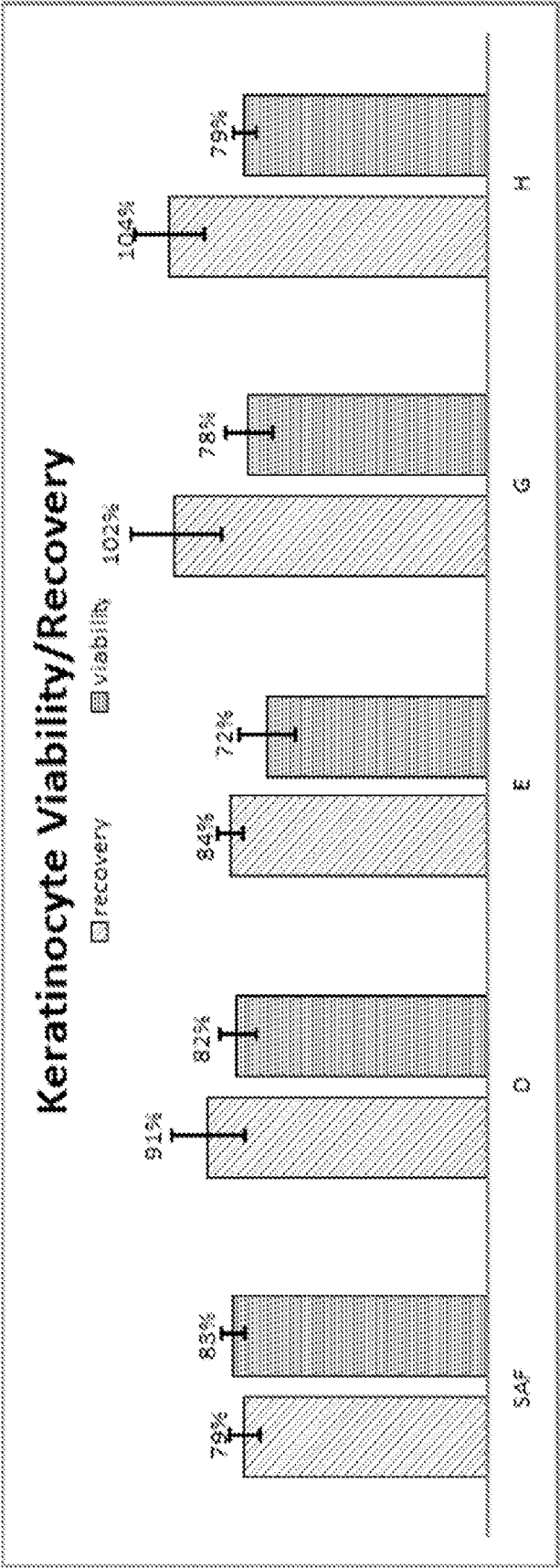
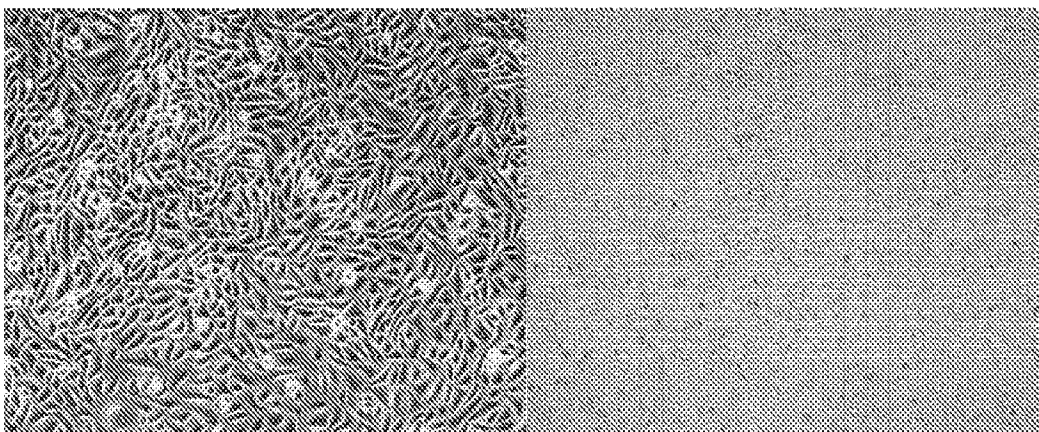


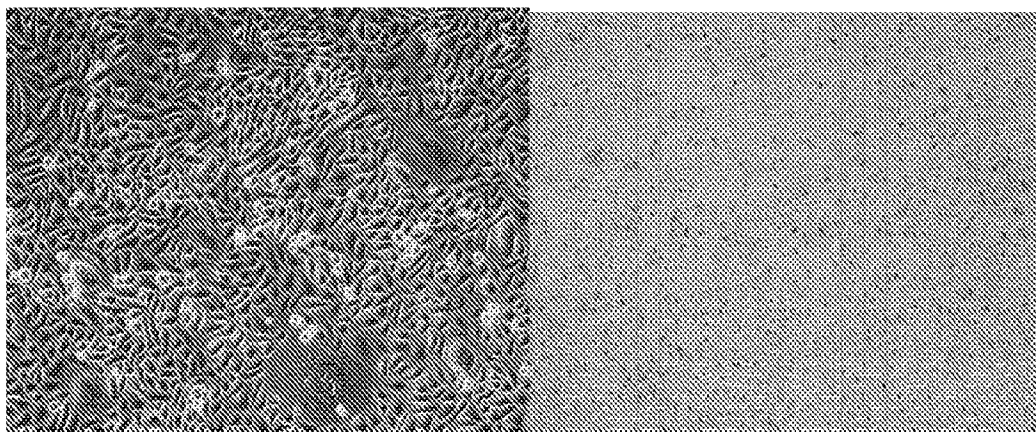
FIG. 6

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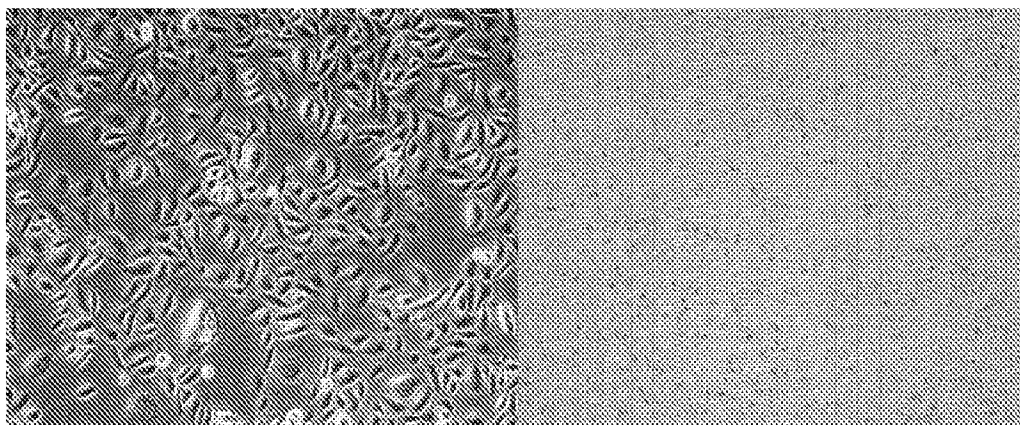
Morphology (100X and 25X) at p4 of PIK41 Keratinocytes Post-Thaw



100X Control Synth-a-Freeze: PIK41 p4 (WCB) 2H Confluence 25X



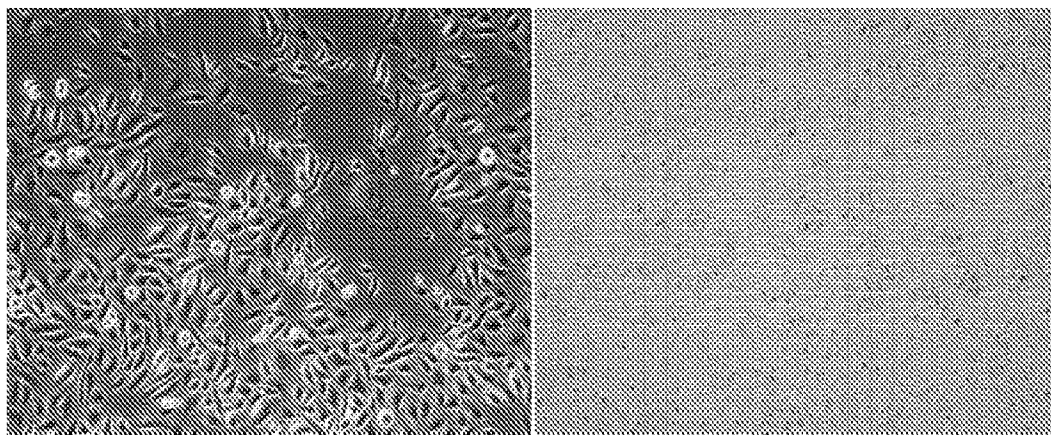
100X Formula D: PIK41 p4 (WCB) 2H Confluence 25X



100X Formula E: PIK41 p4 (WCB) 2H Confluence 25X

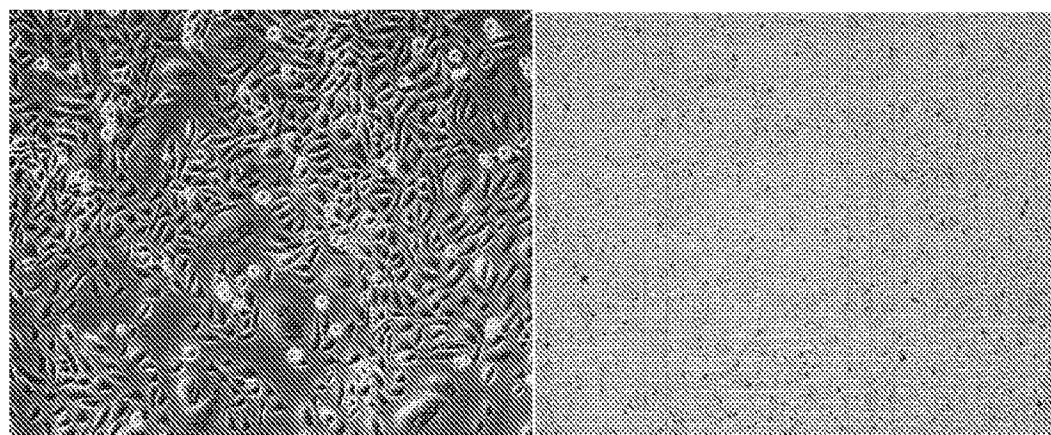
FIG. 7A

Morphology (100X and 25X) at p4 of PIK41 Keratinocytes Post-Thaw



100X Formula G: PIK41 p4 (WCB) 2H Confluence

25X



100X Formula H: PIK41 p4 (WCB) 2H Confluence

25X

FIG. 7B

Harvest Results at p5 of the Cryopreservation Medium Formulations

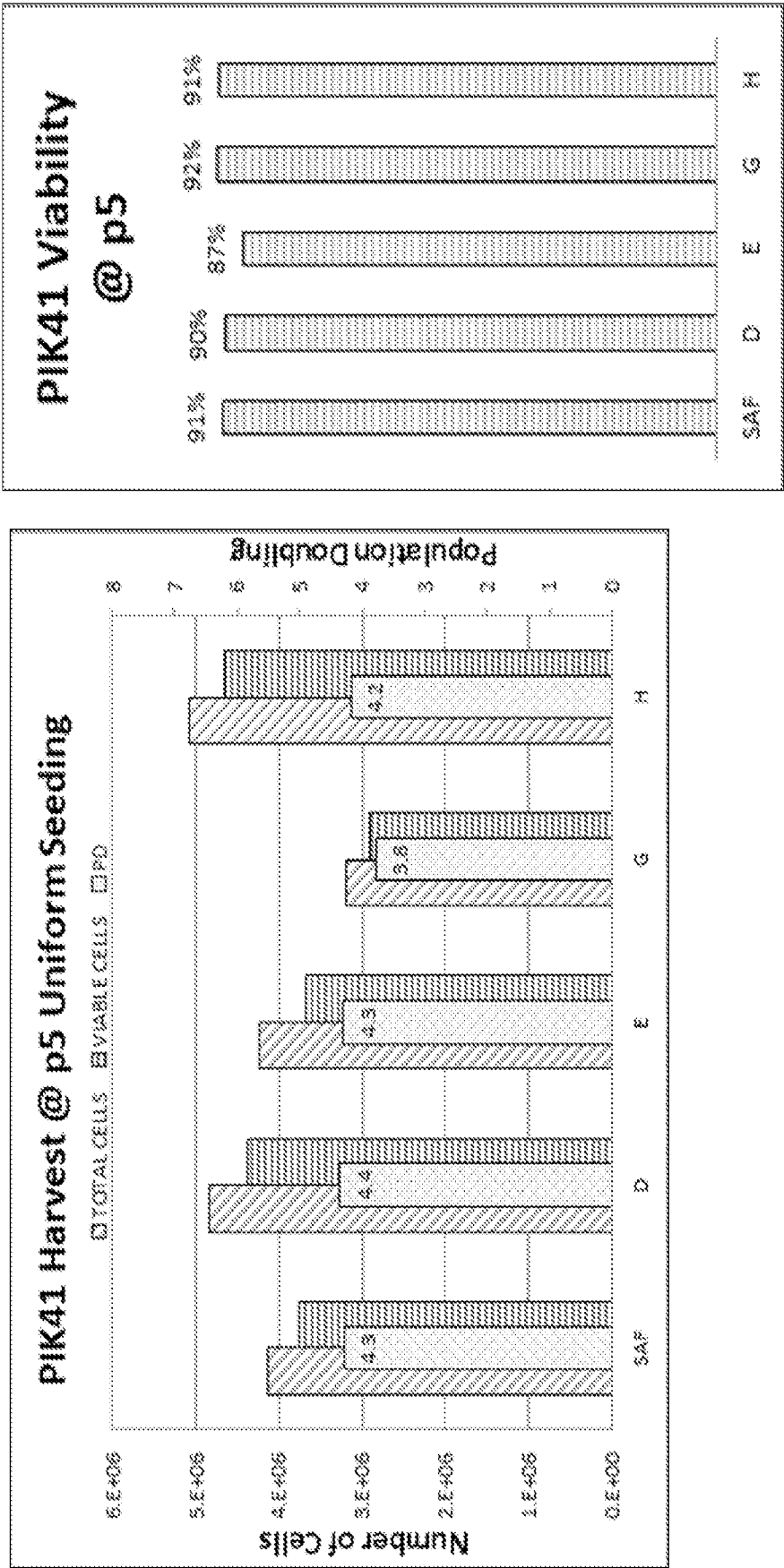


FIG. 8

PIK41 Keratinocyte Thaw Results with LDH values

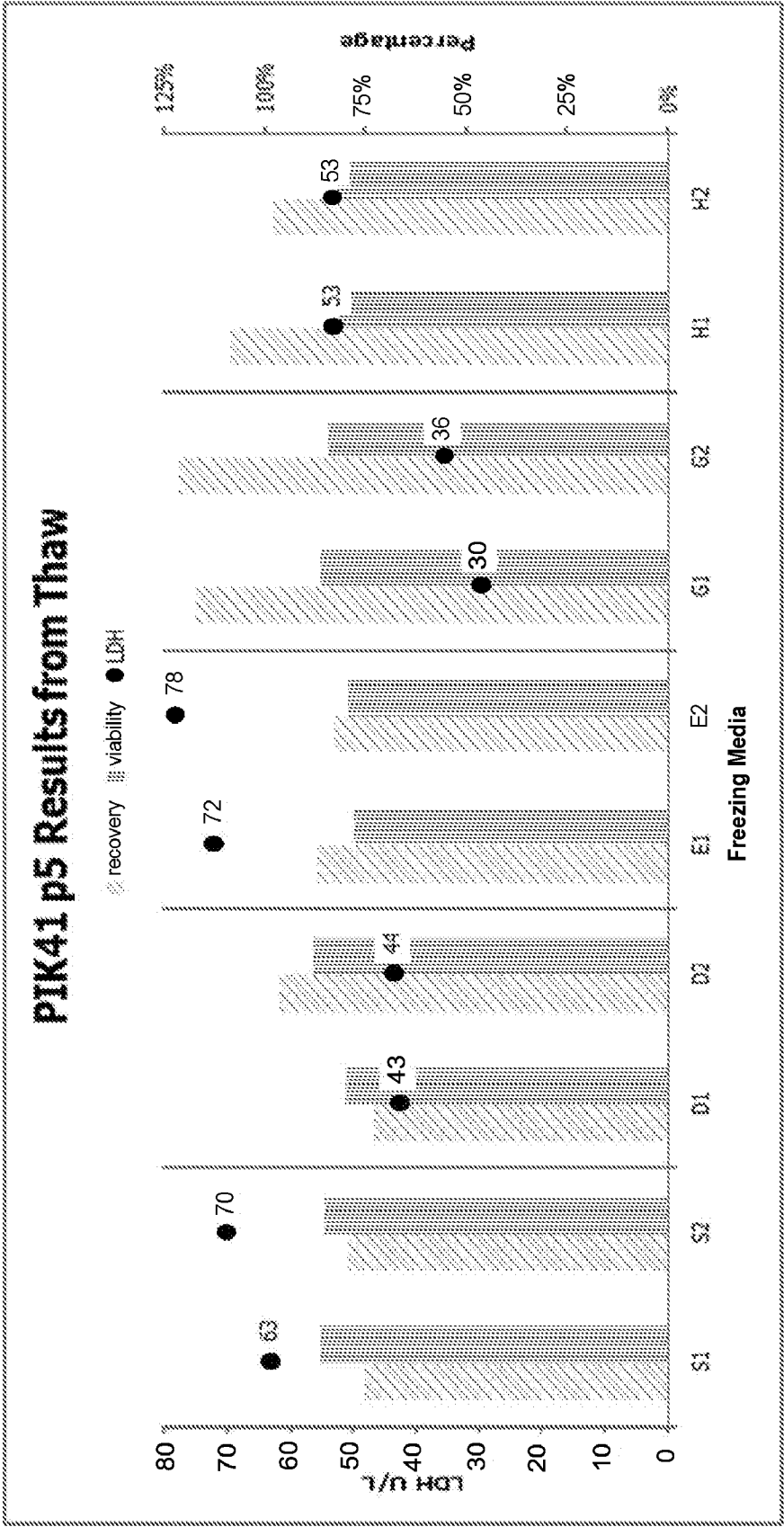


FIG. 9

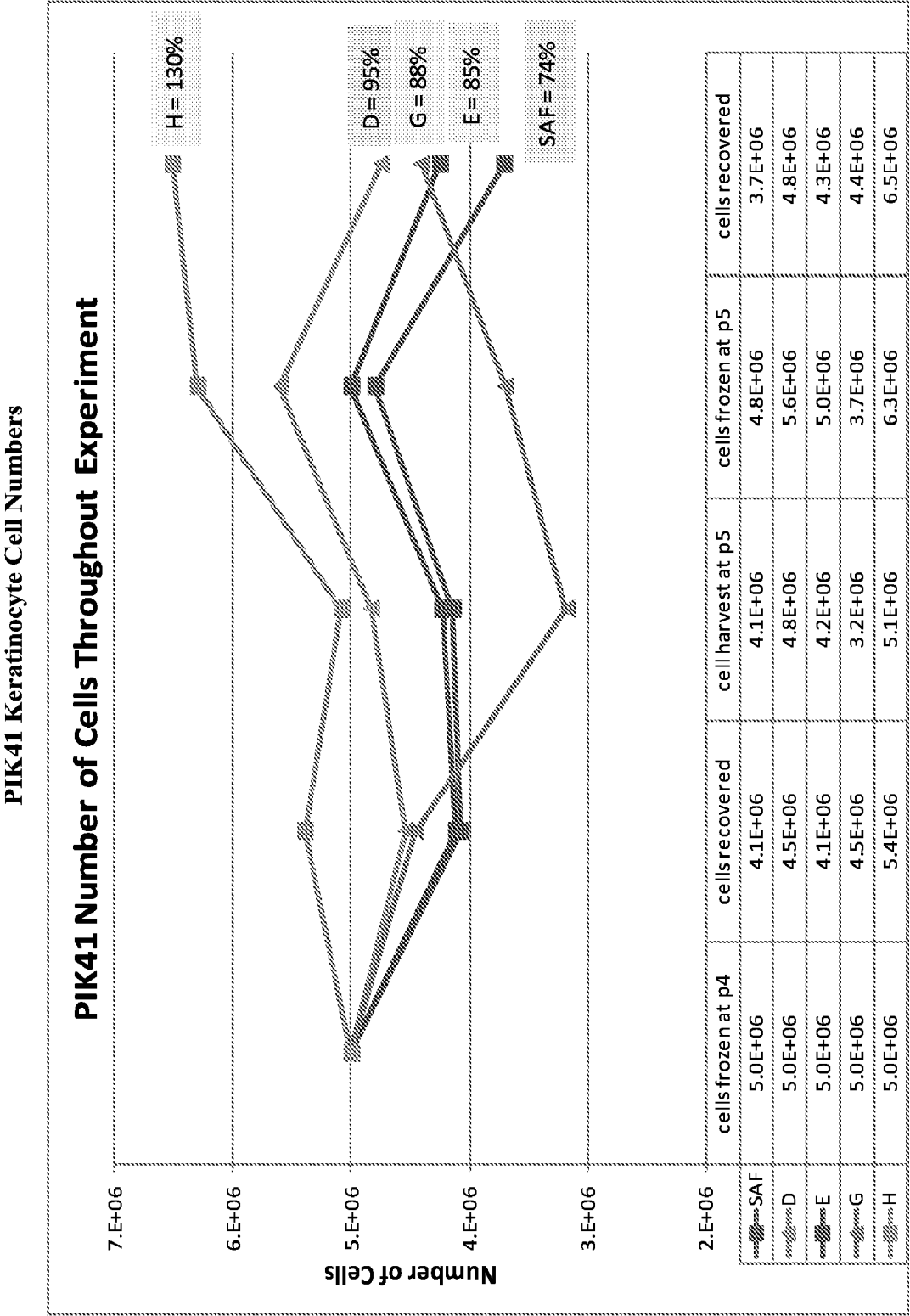
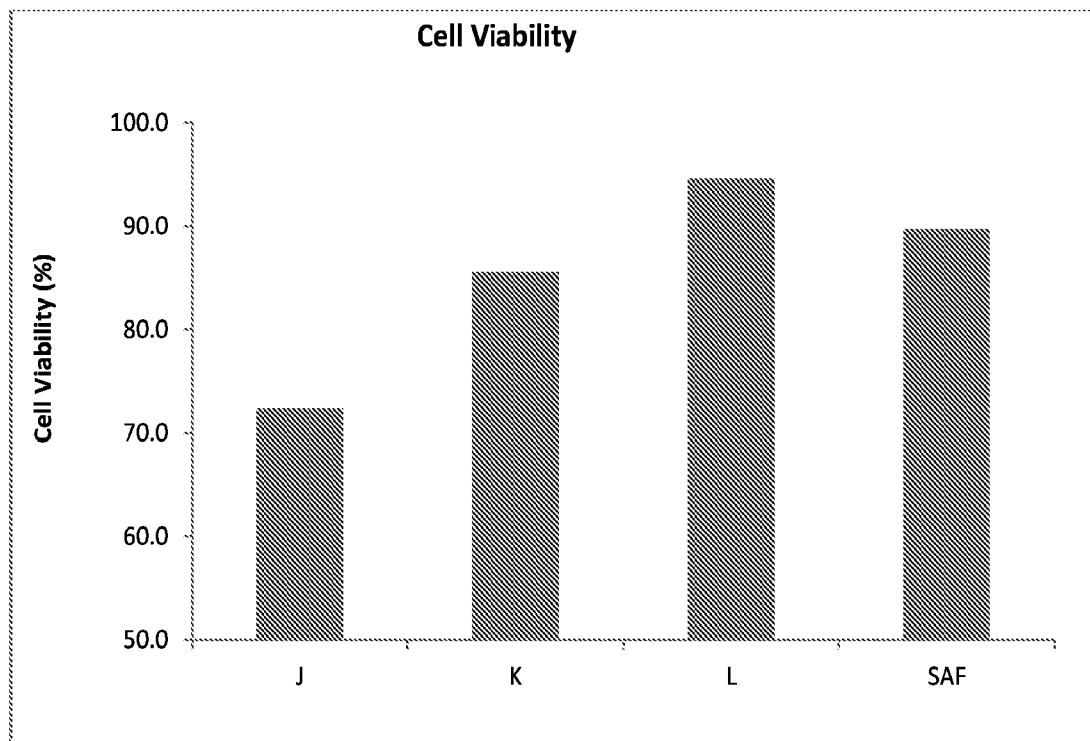
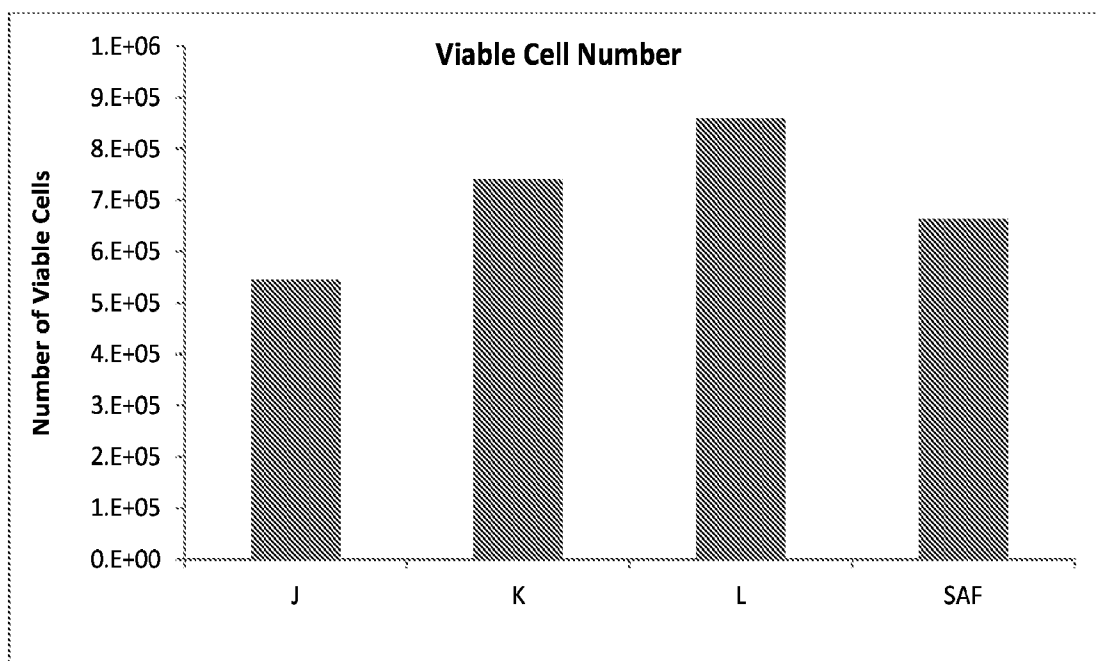
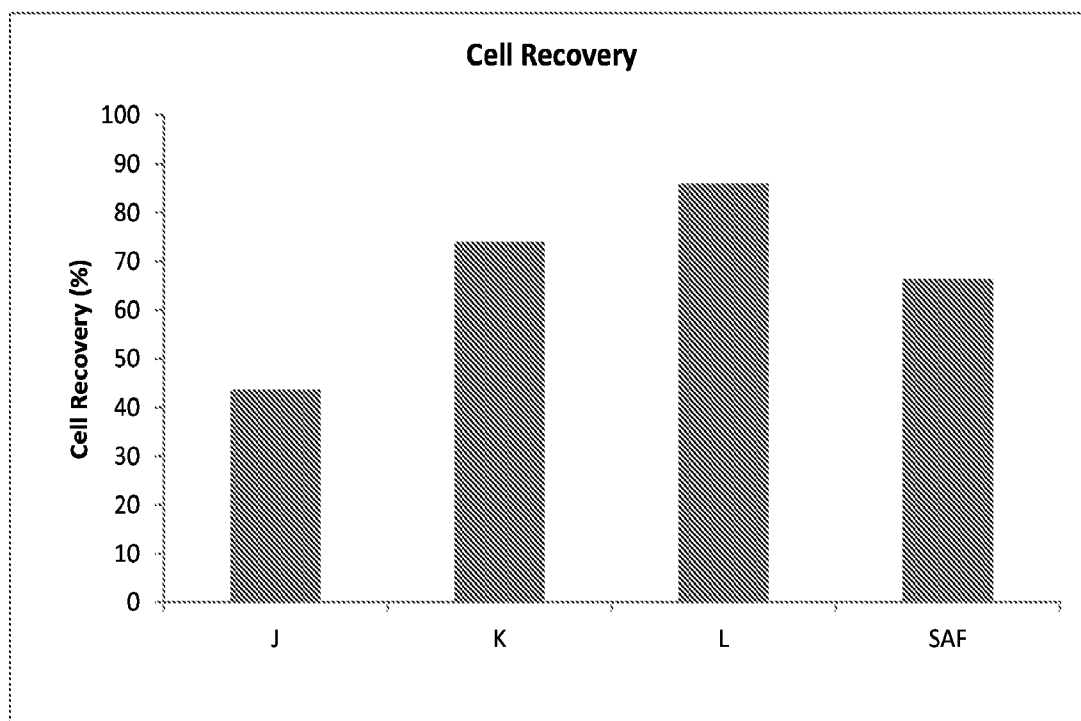
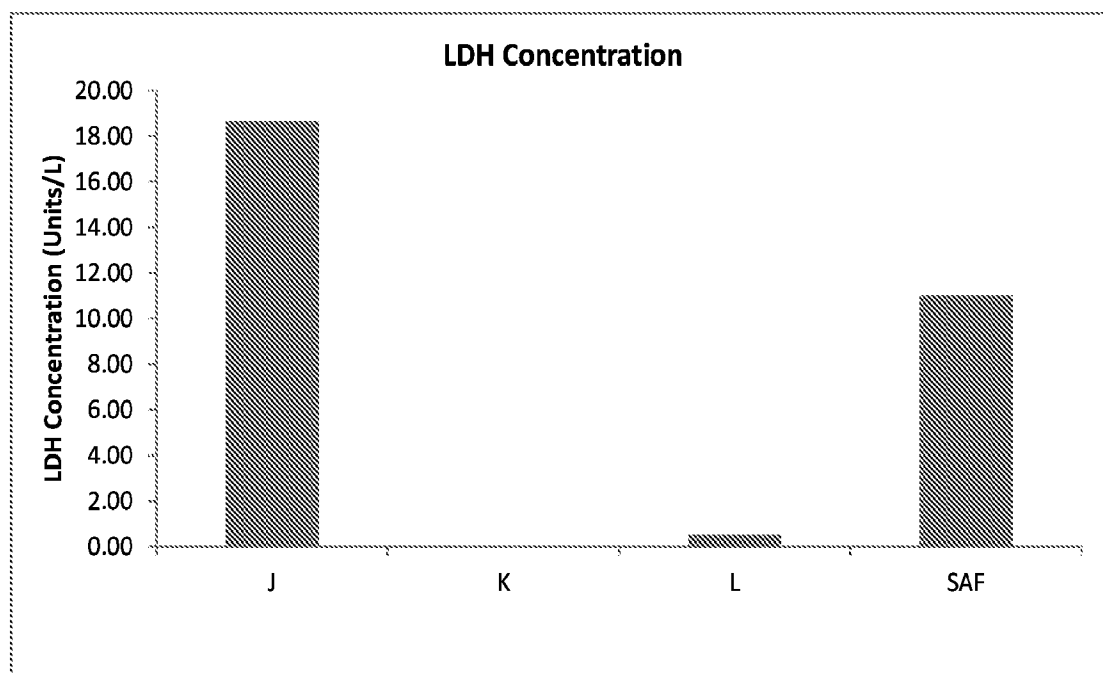


FIG. 10

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**FIG. 11****FIG. 12**

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**FIG. 13****FIG. 14**

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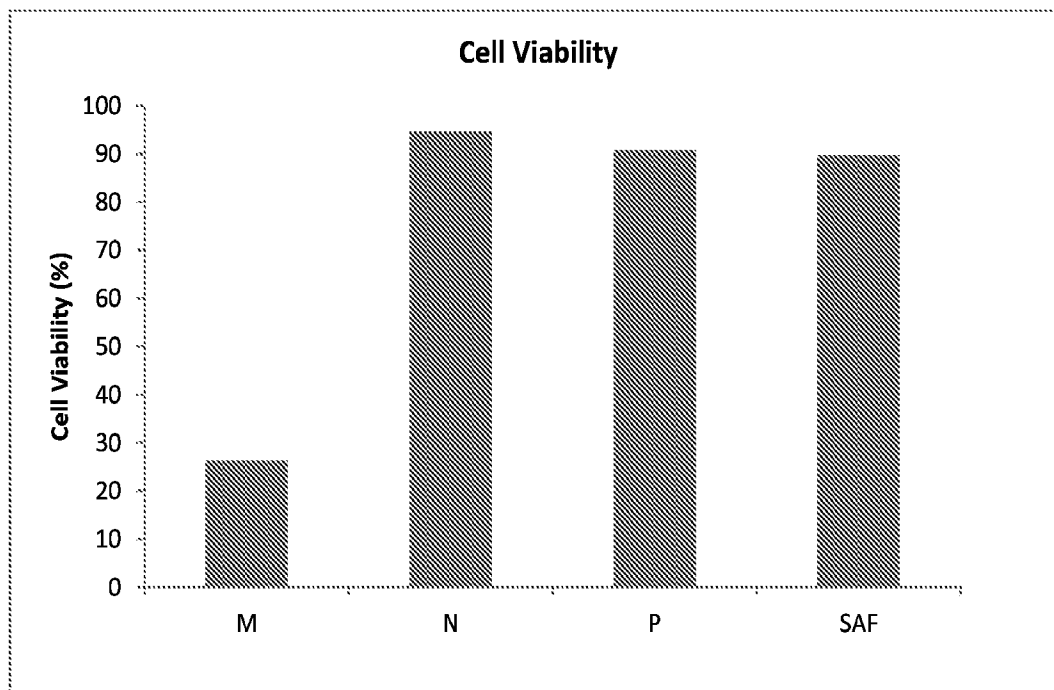


FIG. 15

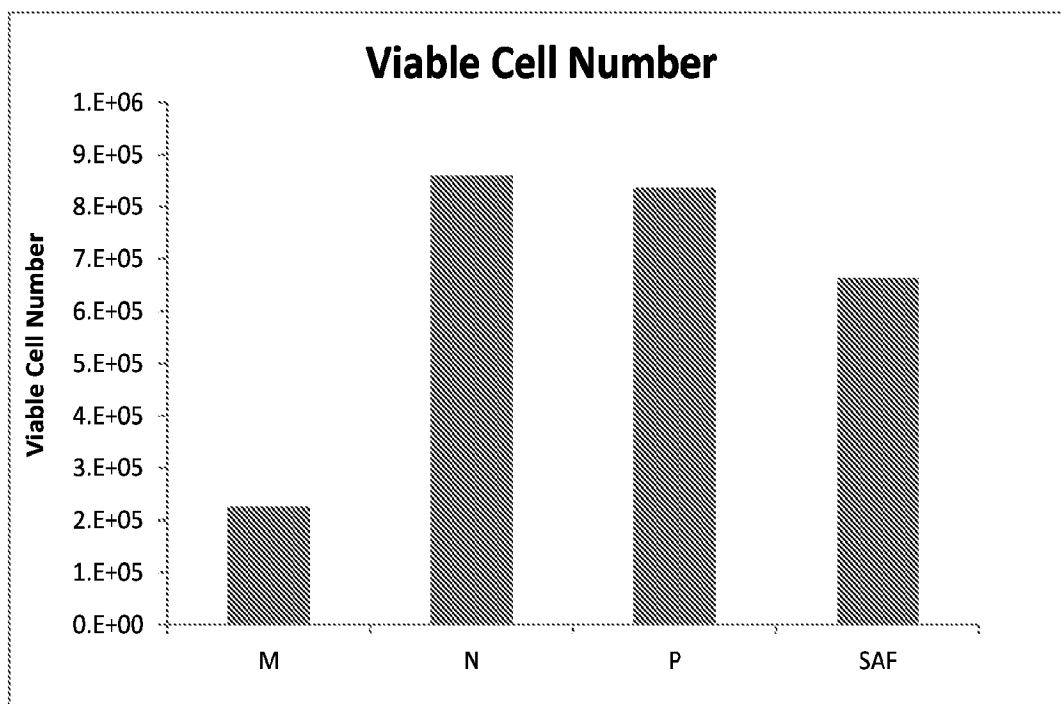
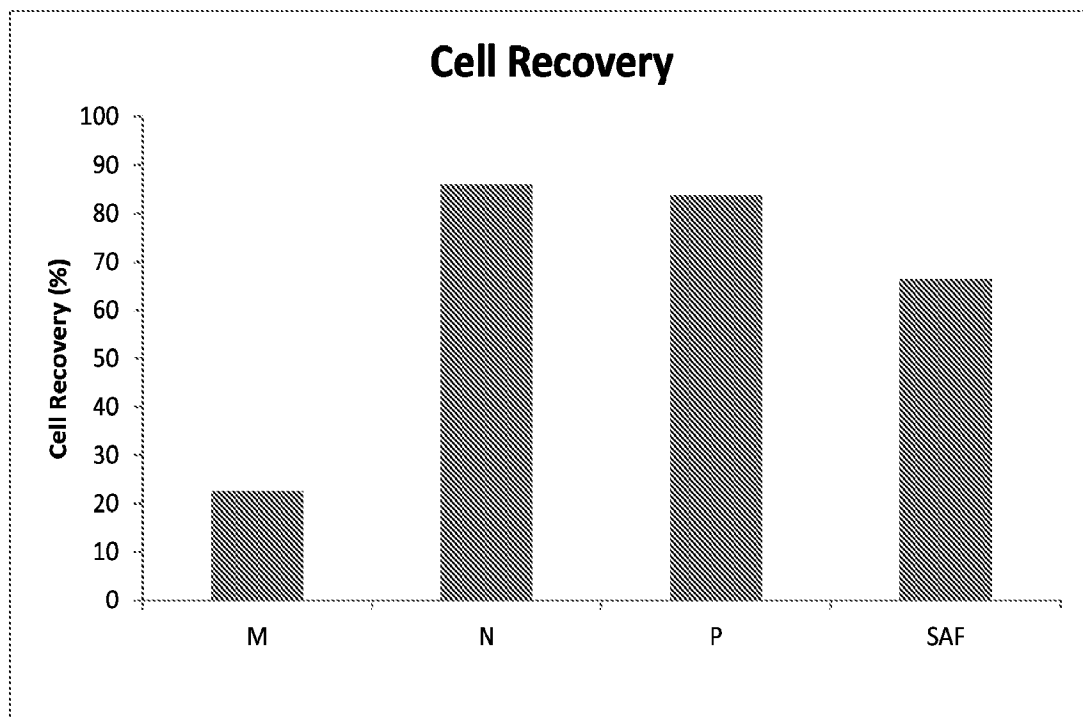
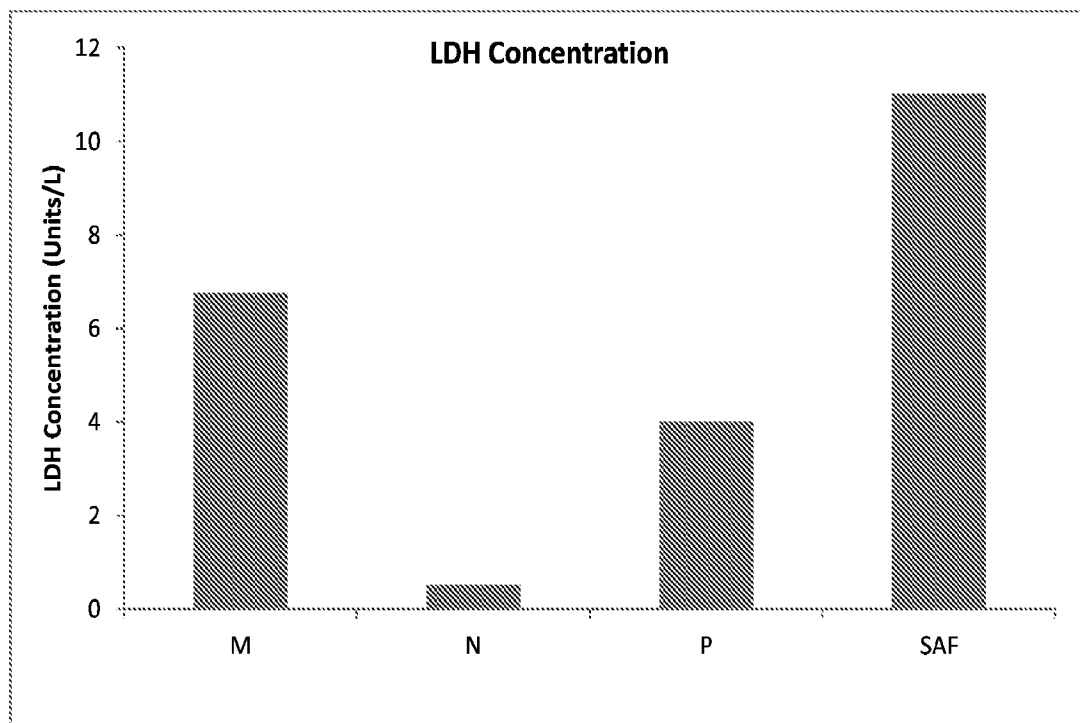
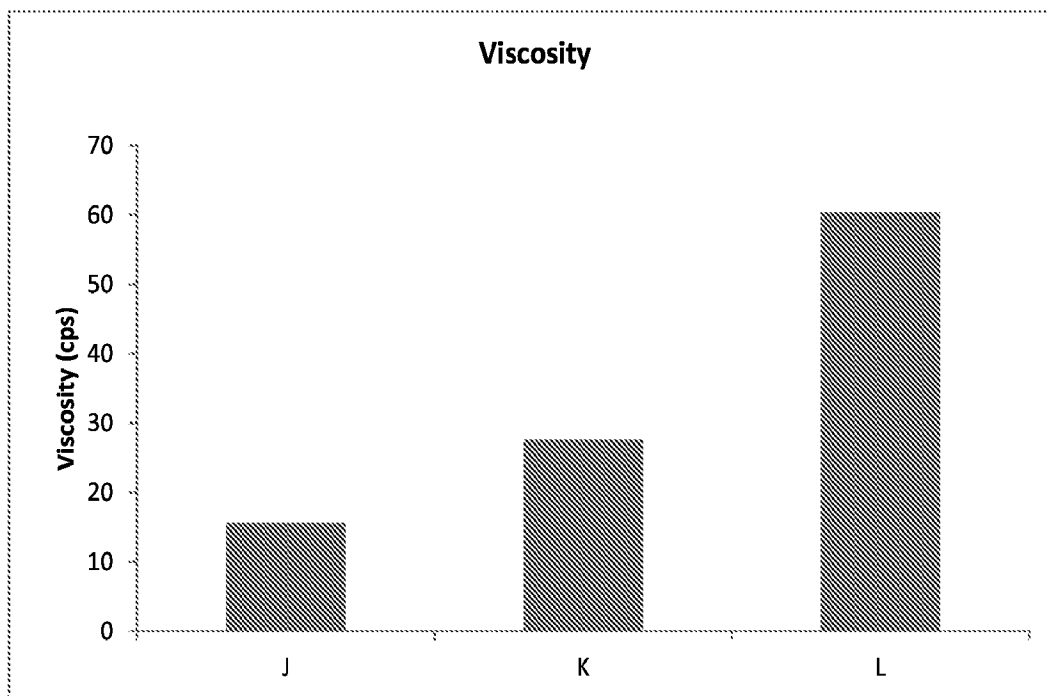
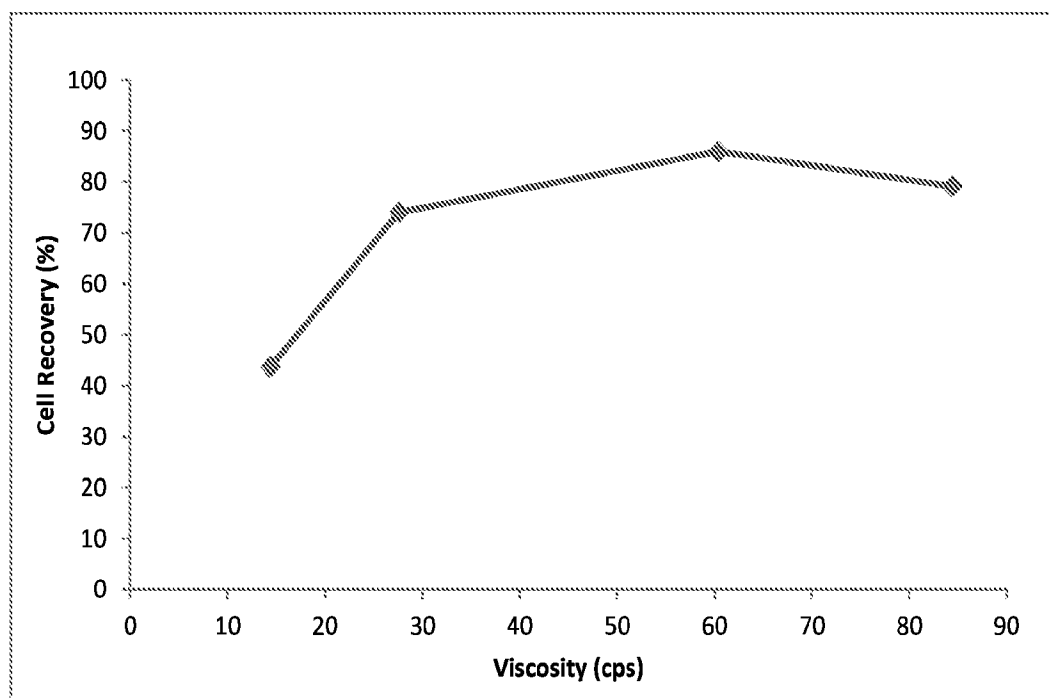


FIG. 16

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**FIG. 17****FIG. 18**

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**FIG. 19****FIG. 20**

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/023587

A. CLASSIFICATION OF SUBJECT MATTER
 INV. A01N1/02
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Y	WO 2007/009285 A1 (ETH ZUERICH [CH]; FISCHER RENE [CH]) 25 January 2007 (2007-01-25) abstract page 2, line 10 - line 25 page 3, line 8 - line 20 page 2, line 28 - line 31 figure 1 example 1	21,24, 26-39, 42,44-55 1-73
X Y	US 2012/128641 A1 (AUSTEN JR WILLIAM G [US]) 24 May 2012 (2012-05-24) abstract paragraphs [0004], [0006], [0033] examples 1-3 figures	21-27 1-73
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Date of mailing of the international search report

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Molina de Alba, José

INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Y	US 2009/017438 A1 (ROY JOSEE [US] ET AL) 15 January 2009 (2009-01-15) abstract paragraphs [0018], [0020], [0023]; claim 1 paragraphs [0044], [0058] -----	1-73

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International application No

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