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Cell Culture Media Containing Combinations of Proteins

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of US provisional patent application No. 61,154,204, filed February 20, 2009, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

BACKGROUND OF THE INVENTION

Cell culture techniques allow animal or plant cells that are removed from tissues to grow when supplied with the appropriate nutrients and conditions. The cells are capable of dividing and can continue to grow until limited by culture variables such as nutrient depletion or toxic buildup (Butler, M. & Jenkins, H., "Nutritional aspects of growth of animal cells in culture," J of Biotechnol. (1989) 12: 97-110). Cell culture techniques have a number of applications including investigation of the normal physiology or biochemistry of cells (Balaban, B. & Urman, B., "Embryo culture as a diagnostic tool." Reprod. Biomed. Online (2003) 7(6): 671-82), testing the effect of various chemical compounds or drugs on specific cell types (Farkas, D. & Tannenbaum, S.R., "In vitro methods to study chemically-induced hepatotoxicity: a literature review," Curr. Drug Metab. (2005) 6(2): 111-25), studying the sequential or parallel combination of various cell types to generate artificial tissues (Wang et al., "Cartilage tissue engineering with silk scaffolds and human articular chondrocytes," Biomaterials (2006)), and synthesizing valuable biologics from large scale cell cultures (Zeilinger et al., "Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions," Altern. Lab Anim. (2002) 30(5): 525-38). Cell culture techniques have also been used for in vitro fertilization (Blake et al., "Protein supplementation of human IVF culture media," J Assist. Reprod. Genet. (2002) 19(3): 137-43; Bungum et al., "Recombinant human albumin as protein source in culture media used

for IVF: a prospective randomized study," Reprod. Biomed. Online (2002) 4(3): 233-6), stem cell research (Conley et al., "Derivation, propagation and differentiation of human embryonic stem cells," Int. J Biochem. Cell Biol. (2004) 36(4): 555-67), vaccine production (Chuang et al., "Pharmaceutical strategies utilizing recombinant human albumin," Pharm. Res. (2002) 19(5): 569-77; GlaxoSmithKline, HAVRIX® (Hepatitis A Vaccine, Inactivated) - Prescribing Information (2005), available at http://us.gsk.com/products/assets/us_havrix.pdf; Innis et al., "Protection against hepatitis A by an inactivated vaccine," JAMA (1994) 271(17): 1328-34; Merck, PROQUAD® - Measles, Mumps, Rubella, and Varicella (Oka/Merck) Virus Vaccine Live - Prescribing Information (2005), available at http://www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf; Litwin, J., "The growth of Vero cells in suspension as cell-aggregates in serum-free media," Cytotechnology (1992) 10(2): 169-74), tissue engineering including artificial skin (Atala, A., "Future perspectives in reconstructive surgery using tissue engineering," Urol. Clin. North Am. (1999) 26(1): 157-65. ix-x; Sher. et al., "Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation," J Biol. Chem. (2006) 281(8): 5178-87) and organs (Neronov et al., "Integrity of endothelium in cryopreserved human cornea," Cryo Letters (2005) 26(2): 131-6; Han, et al., "Interleukin-1 alpha-induced proteolytic activation of metalloproteinase-9 by human skin." Surgery (2005) 138(5): 932-9) and gene and cell therapy (Chadd. H.E. & Chamow, S.M., "Therapeutic antibody expression technology." Curr. Opin. Biotechnol. (2001) 12(2): 188-94).

Biologics encompass a broad range of cell products, and include specific proteins or viruses that require animal cells for propagation. For example, therapeutic proteins such as monoclonal antibodies can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures (Dewar et al., "Industrial implementation of in vitro production of monoclonal antibodies, Ilar J (2005) 46(3): 307-13). The number of such commercially valuable biologics has increased rapidly over the last decade and has led to the present widespread interest in mammalian cell culture technology (Mizrahi, A., "Biologicals produced from animal cells in culture – an overview," Biotechnol. Adv. (1988) 6(2): 207-20).

The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The need for cell culture, especially at large scale, became apparent with the need for viral vaccines. Major epidemics of polio in the 1940s and 1950s promoted efforts to develop an effective vaccine. In 1949, it was shown that poliovirus could be grown in cultures of human cells, which

led to considerable interest in the development of large quantities of the polio vaccine using cell culture (Ligon, B.L., "Thomas Huckle Weller MD: Nobel Laureate and research pioneer in poliomyelitis, Varicella-zoster virus, cytomegalovirus, rubella, and other infectious diseases," Semin. Pediatr. Infect. Dis. (2002) 13(1): 55-63). The polio vaccine, produced from de-activated virus, became one of the first commercial products of cultured animal cells (Furesz, J., "Developments in the production and quality control of poliovirus vaccines – Historical perspectives," Biologicals (2006)).

Due to the safety and ethical considerations associated with the use of animal-derived cell culture media components, efforts have been made to provide alternative sources for cell culture media and media components.

Published PCT Appl. No. WO 2007/002762, which was based on U.S. Provisional Appl. No. 60/694,236, filed June 28, 2006, relates to recombinant production of components of cell culture media using plant cells, and cell culture media containing such recombinant proteins. The entire contents of this application are incorporated herein by reference.

SUMMARY OF THE INVENTION

Accordingly, it is an objective of the invention to provide cell culture media or media supplements containing combinations of protein components, which when used in cell culture, result in improved growth and/or productivity of cultured cells.

One embodiment of the invention is a cell culture media including a cell culture media base, and two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

Yet another embodiment of the invention is a cell culture media including a cell culture media base, albumin, and at least one additional protein selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor,

glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the growth factors that may be provided in the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

Another embodiment of the invention is a cell culture media including a cell culture media base, lactoferrin, and at least one additional protein selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to further embodiments, a preferred combination of proteins that may be provided in the cell culture media includes lactoferrin and albumin.

Another embodiment of the invention provides methods for producing supplemented cell culture media, including providing a cell culture media base; providing two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-

lactalbumin; and combining the two or more proteins with the cell culture media base to form the supplemented cell culture media.

According to further embodiments, the proteins used in the methods for producing cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be provided in the methods of producing the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of producing the cell culture media includes lactoferrin and albumin.

Another embodiment of the invention provides a method for culturing cells, including providing a cell culture media base; supplementing the cell culture media base with two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin to form a supplemented cell culture media; and introducing cells to be cultured into the supplemented cell culture media. The cells grown in the supplemented cell culture media exhibit improved growth characteristics as compared to cells grown in an unsupplemented cell culture media.

According to further embodiments, the proteins used in the methods for culturing cells are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for culturing cells are plant-

produced heterologous proteins, preferably two or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for culturing cells are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be used in the methods for culturing cells are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, by culturing the cultured cell in the cell culture media of the present invention

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and a transferrin related protein: wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a supplement comprising: a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100,000, to about 1 to 1,000.

In one aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein. In aspect of this embodiment, the heat shock protein is a rice heat shock protein. In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein. In another aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469.

In another aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt/wt HSP70.

In another aspect of any of the claimed supplements, the transferrin related protein is lactoferrin. In another aspect of any of the claimed supplements, the transferrin related protein is transferrin. In one aspect, of any of the claimed supplements the lactoferrin is human. In one aspect, of any of the claimed supplements the transferrin is human. In one aspect, of any of the claimed supplements the lactoferrin is recombinant. In one aspect, of any of the claimed supplements the lactoferrin is derived from plasma. In one aspect, of any of the claimed supplements the transferrin is recombinant. In one aspect, of any of the claimed supplements the transferrin is derived from plasma. In one aspect, of any of the claimed supplements the IGF-1 is human. In one aspect, of any of the claimed supplements the IGF-1 is recombinant.

In another aspect of any of the claimed supplements, the ratio of transferrin to albumin about 1 to 50, to about 1 to 200. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 75, to about 1 to 180. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 80, to about 1 to 120. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 1000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 2000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 3000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 4000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 5000.

In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0.5, to about 1 to 2. In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises: a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the

bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i)

produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and

wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises: a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the cell culture medium wherein the supplement comprises: a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein

is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 5000. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In one aspect of any of these methods, the supplement further comprises insulin. In one aspect of any of these methods, the supplement further comprises IGF-1. In another aspect, the supplement further comprises selenium and ethanolamine.

In another aspect of any of the claimed methods, the cells are tissue culture cells. In another aspect, the cells are CHO cells. In another aspect the cells are hybridoma cells. In another aspect the cells are vero cells. In another aspect the cells are sorted by flow cytometry. In another aspect the cells are primary cells. In one aspect, the primary cells are embryonic stem cells. In another aspect the primary cells are B-cell derived. In another aspect the cells the primary cells are T-cell derived. In another aspect the cells are B-cells or B-cell derived. In another aspect the cells are T-cells or T-cell derived. In another aspect the cells. In another aspect the cells are isolated by a micro fluidic device. In another aspect the cells are isolated by single-cell subcloning.

In one aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt / wt of a heat shock protein. In aspect of this embodiment, the heat shock protein is a rice heat shock protein. In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein. In another

aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469.

In another aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt /wt HSP70.

In another aspect of any of the claimed methods, the transferrin related protein is lactoferrin. In another aspect of any of the claimed methods the transferrin related protein is transferrin. In one aspect, of any of the claimed methods the lactoferrin is human. In one aspect, of any of the claimed methods the transferrin is human. In one aspect, of any of the claimed methods the lactoferrin is recombinant. In one aspect, of any of the claimed methods the transferrin is recombinant.

In another aspect of any of the claimed methods, the ratio of transferrin to albumin is about 1 to 50. to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 75. to about 1 to 180. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 80, to about 1 to 120. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 120, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 200, to about 1 to 1000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 1000, to about 1 to 2000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 2000, to about 1 to 3000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 3000, to about 1 to 4000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 4000, to about 1 to 5000.

In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.5, to about 1 to 2. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 100 mg /L and 400 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 400 mg /L and 800 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 800 mg /L and 1000mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 1000 mg /L and 2000 mg/ L.

In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 1ug /L and 50 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 2 ug /L and 10 ug/ L In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 3 ug /L and 8 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 4 ug /L and 6 ug/ L.

In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 100 mg /L and 2000 mg/ L. In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 200 mg /L and 1000 mg/ L.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 30 % compared to the summed effect of recombinant albumin and transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 40 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 50 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 60 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 70 % compared to the summed effect of recombinant albumin or transferrin related protein alone when measured under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 80 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 90 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 100 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 150 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 200 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

Other novel features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

BRIEF DESCRIPTION OF THE FIGURES

For a more complete understanding of the present invention, the needs satisfied thereby, and the objects, features, and advantages thereof, reference now is made to the following description taken in connection with the accompanying drawings.

Figure 1 is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

Figure 2 is a plasmid map of the 5,817 bp plasmid, API164 (GT1-Lac), showing an expression cassette for human lactoferrin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human lactoferrin, a Nos terminator and a kanamycin resistance selectable marker.

Figure 3 shows the results of a SDS-PAGE analysis for the expression of recombinant human lactoferrin. Total proteins from rice seed extracts were suspended in Laemli sample buffer, run on a gradient gel and stained with Coomassie blue. Lane 1 is the molecular weight marker. Lanes 3 to 6 are purified human derived lactoferrin (Sigma Chemical, USA). Lanes 8 to 13 are single seed extracts from homozygous independent transgenic rice lines and lane 14 is a seed extract from non-transformed rice variety Taipei 309.

Figure 4 is a stable expression of recombinant human lactoferrin in transgenic rice grains from R_2 through R_{10} generations. Total soluble proteins from 1 g of brown rice flour was extracted with 40 ml of extraction buffer and clarified by centrifugation. The extract was analyzed via ELISA. Extraction was repeated three times and standard deviation is shown as an error bar.

Figure 5 shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

Figure 6 is a plasmid map of the 5,496 bp plasmid, API504 (GT1-HSA), showing an expression cassette for human albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

Figure 7 is a plasmid map of the 12,388 bp plasmid, API508 (JH/GT1-HSA), showing an expression cassette for human serum albumin (HSA) and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

Figure 8 Shows a comparison by HPLC size exclusion chromatography of recombinant HSA produced from rice compared to other sources of albumin and methods of purification. **Figure 8A** shows the chromatogram for a serum derived (non-recombinant HSA). **Figure 8B** shows the chromatogram for a rice recombinant HSA (Cellastim P0107) made using the "old process" B000 for purification. **Figure 8C** shows the chromatogram for a rice recombinant HSA (Cellastim P0171) made using the "new process" B0000C for purification. **Figure 8D** shows an overlay of the chromatograms for the serum derived albumin (1A; dotted line) and Cellastim HSA prepared using the new process ((1C; solid line). **Figure 8E** shows an overlay of the chromatograms for HSA prepared using the old process B000 (Cellastim P0107) (1B; dotted line) and HSA prepared using the new process B0000C (Cellastim P0171) (1C; solid line).

Figure 9 Shows a comparison by SDS PAGE analysis of recombinant albumin produced from rice compared to other sources of HSA and methods of purification. **Figure 9A** shows a comparison of Cellastim P0171albumin and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **Figure 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3. and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

Figure 10: Shows a comparison of the effects of yeast recombinant HSA (Cellprime), human plasma derived HSA, (Seracare) and plant recombinant HSA (Cellastim P0171) with respect to their ability to promote cell growth and viability. (**Figure 10A**). **Figure 10B** shows a comparison of the endotoxin levels in batches of albumin produced using the old (B000) and new processes (B0000C) for recombinant albumin production. **Figure 10C** shows a comparison of cell growth and viability of cells grown in the presence of the Cellastim HSA produced using the old (B000) and new processes (B0000C) for recombinant albumin production.

Figure 11: Shows a western blot of two independent experiments showing that plant derived recombinant HSA comprises proteins that can be selectively concentrated by an ATP affinity

column (Lanes 4 & 9) and react against an anti-HSP antibody. Lanes 3 and 7 show 10 ug of starting material; Lanes 4 and 9 show the fractions after elution and concentration from the ATP affinity column; Lanes 5 and 8 show the wash through after elution with ATP, and lane 10 is a hsp positive control. (See Example 9 for details).

Figure 12: Shows a comparison of the cell growth and viability effect of Cellastim recombinant albumin after passing the albumin produced using the new process over an ATP affinity column to remove heat shock proteins. (See text for details).

Figure 13: Shows a comparison of the effect of 1 g/L recombinant plant derived human serum albumin (rHSA) in combination with transferrin (pTF) compared to the same concentration of plasma derived human serum albumin (pHSA) in combination with transferrin (pTF) on the production of antibody by hybridoma cells in culture.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description. Unless otherwise indicated, all terms used herein have the meanings given below, or are generally consistent with same meaning that the terms have to those skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds. (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands, for definitions and terms of the art.

The term "**about**" or "**approximately**" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviations, per practice in the art. Alternatively, "about" with respect to the compositions can mean plus or minus a range of up to 20%, preferably up to 10%, more preferably up to 5%.

The term **"antigen-binding fragment"** refers to a polypeptide portion of an immunoglobulin or antibody that binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). Binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies.

The term "**apoptosis**" ("normal" or "programmed" cell death) refers to the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine dependent tissue atrophy. Apoptosis may also be triggered in cells grown under tissue culture conditions in response to stress. Cells undergoing apoptosis show characteristic morphological and biochemical features, which can be readily measured and quantified. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed "secondary necrosis".

As used herein, the terms "**cell**," "**cells**," "**cell line**," "**host cell**," and "**host cells**," are used interchangeably and, encompass plant, and animal cells and include invertebrate, non-mammalian vertebrate and mammalian cells. All such designations include cell populations and progeny. Thus, the terms "transformants" and "transfectants" include the primary subject cell and cell lines derived therefrom without regard for the number of transfers. Exemplary non-mammalian vertebrate cells include, for example, avian cells, reptilian cells and amphibian cells. Exemplary invertebrate cells include, but are not limited to, insect cells such as, for example, caterpillar (Spodoptera frugiperda) cells, mosquito (Aedes aegypti) cells, fruitfly (Drosophila melanogaster) cells, Schneider cells, and Bombyx mori cells. See, e.g., Luckow et al., Bio/Technology 6:47-55 (1988). The cells may be differentiated, partially differentiated or

undifferentiated, e.g. stem cells, including embryonic stem cells and pluripotent stem cells. Additionally tissue samples derived from organs or organ systems may be used according to the invention. Exemplary mammalian cells include, for example, cells derived from human, non-human primate, cat, dog, sheep, goat, cow, horse, pig, rabbit, rodents including mouse, hamster, rat and guinea pig and any derivatives and progenies thereof.

The terms "**cell culture**," or "**tissue culture**" refer to cells grown in suspension or grown adhered to a variety of surfaces or substrates in vessels such as roller bottles, tissue culture flasks, dishes, multi-well plates and the like. Large scale approaches, such as bioreactors, including adherent cells growing attached to microcarriers in stirred fermentors, are also encompassed by the term "cell culture." Moreover, it is possible not only to culture contact-dependent cells, but also to use suspension culture techniques in the methods of the claimed invention. Exemplary microcarriers include, for example, dextran, collagen, plastic, gelatin and cellulose and others as described in Butler, Spier & Griffiths, Animal cell Biotechnology 3:283-303 (1988). Porous carriers, such as, for example, Cytoline™ or Cytopore™, as well as dextran-based carriers, such as DEAE-dextran (Cytodex 1™ quaternary amine-coated dextran (Cytodex™) or gelatin-based carriers, such as gelatin-coated dextran (Cytodex 3™) may also be used. Cell culture procedures for both large and small-scale production of proteins are encompassed by the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, with or without microcarriers, and operated alternatively in a batch, fed-batch, or perfusion mode.

The terms "**cell culture medium**," "**cell culture media**," and "**culture medium**" refer to the solutions used for growing, storing, handling and maintaining cells and cell lines. Such solutions generally include various factors necessary for cell attachment, growth, and maintenance of the cellular environment. For example, a typical solution may include a basal media formulation, various supplements depending on the cell type and, occasionally, antibiotics. In some embodiments, a solution may include at least one component from one or more of the following categories: 1) an energy source, usually in the form of a carbohydrate such as glucose; 2) all essential amino acids, and usually the basic set of twenty amino acids plus cystine; 3) vitamins and/or other organic compounds required at low concentrations; 4) free fatty acids; and 5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The solution may optionally be supplemented with one or more components from any of the following categories: 1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor; 2) salts and buffers as, for example, calcium, magnesium, phosphate, Tris, HEPES, and sodium bicarbonate; 3) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and 4) protein and tissue hydrolysates. In general, any suitable cell culture medium may be used. The medium may be comprised of serum, e.g. fetal bovine serum, calf serum or the like. Alternatively, the medium may be serum free, animal free, or protein free.

The term "**cell culture media component**" or "cell culture component" refers to any heterologous proteins used as a supplement for cell culture media.

The term "**cell culture media ingredient**" includes Cell culture media components, proteins, peptides, hormones, carbohydrates, amino acids, lipids, vitamins, antibiotics, organic and inorganic salts.

The term "**cell culture media supplement**" refers to a combination of one or multiple cell culture media components, with or without other ingredients, for addition to cell culture media.

The term "**cell culture media base**" or "**basal media**" refers to a cell culture media that may contain, for example, any or all of the following components: proteins, peptides, lipids, carbohydrates, amino acids, organic and/or inorganic salts, buffers (e.g., bicarbonate), vitamins, hormones, antibiotics, and pH indicators (e.g., phenol red). Examples of cell culture media bases that can be used in accordance with the present invention include: Dulbecco's Modified Eagle's Medium (DME), Ham's Nutrient Mixtures, MCDB Media, Minimum Essential Medium Eagle, RPMI Media, Ames' Media, BGJb Medium (Fitton-Jackson Modification), Click's Medium, CMRL-1066 Medium, Fischer's Medium, Glascow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), L-15 Medium (Leibovitz), McCoy's 5A Modified Medium, NCTC Medium, Swim's S-77 Medium, Waymouth Medium, and William's Medium E. The cell culture media base may be supplemented using a cell culture media component or a cell culture media supplement.

The term "**cell lineage**" when referring to a stem cell culture refers to all of the stages of the development of a cell type, from the earliest precursor cell to a completely mature cell (i.e. a specialized cell).

The terms "**cell viability**" or "**viability**" refers to relative amounts of living and dead cells, present with a population of cells at any given time. Cell viability may be determined by measuring the relative numbers of living and dead cells in any given sample of the population. Cell viability may also be estimated by measuring the rate of cell proliferation of the entire population which represents the overall balance of the rates of cell growth and cell death. Rates of cell growth may also be directly measured, by counting the number of cells, and by using any number of commercially available cell proliferation assays which directly scores the rate of cell growth.

"**Conditioned medium**" refers to a cell culture medium that is obtained from a culture of a feeder cell on which embryonic stem cells can be cultured and maintained in a pluripotent state. The feeder cell depletes the conditioned medium of some components, but also enriches the medium with cell-derived material, probably including small amounts of growth factors. The term "feeder cell factor" as used herein means the cell-derived material that is released into the conditioned medium by the feeder cell. The cell factor that is released into the cell culture medium is useful in enhancing the growth of embryonic stem cells, or in the maintenance of the embryonic stem cell in a pluripotent state. The feeder cell factor can be identified and purified using techniques that are known to one skilled in the art, and are described herein.

The phrase "**conservative amino acid substitution**" or "**conservative mutation**" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag).

Examples of amino acid groups defined in this manner include: a "charged / polar group," consisting of Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic, or cyclic group," consisting of

Pro, Phe, Tyr and Trp; and an "aliphatic group" consisting of Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys.

Within each group, subgroups can also be identified, for example, the group of charged / polar amino acids can be sub-divided into the sub-groups consisting of the "positively-charged sub-group," consisting of Lys, Arg and His; the negatively-charged sub-group," consisting of Glu and Asp, and the "polar sub-group" consisting of Asn and Gln. The aromatic or cyclic group can be sub-divided into the sub-groups consisting of the "nitrogen ring sub-group," consisting of Pro, His and Trp; and the "phenyl sub-group" consisting of Phe and Tyr. The aliphatic group can be sub-divided into the sub-groups consisting of the "large aliphatic non-polar sub-group," consisting of Val, Leu and Ile; the "aliphatic slightly-polar sub-group," consisting of Met, Ser, Thr and Cys; and the "small-residue sub-group," consisting of Gly and Ala.

Examples of conservative mutations include substitutions of amino acids within the sub-groups above, for example, Lys for Arg and vice versa such that a positive charge can be maintained; Glu for Asp and vice versa such that a negative charge can be maintained: Ser for Thr such that a free -OH can be maintained; and Gln for Asn such that a free -NH_2 can be maintained.

The term "**cytotoxicity**" refers to the cell killing property of a chemical compound (such as a chemical or protein contaminant, detergent, or toxin). In contrast to necrosis and apoptosis, the term cytotoxicity need not necessarily indicate a specific cellular death mechanism.
As used herein, the term "**decrease**" or the related terms "**decreased**," "**reduce**" or "**reduced**" refers to a statistically significant decrease. For the avoidance of doubt, the terms generally refer to at least a 10% decrease in a given parameter, and can encompass at least a 20% decrease, 30% decrease, 40% decrease, 50% decrease, 60% decrease, 70% decrease, 80% decrease, 90% decrease, 95% decrease, 97% decrease, 99% or even a 100% decrease (i.e., the measured parameter is at zero).

As used herein, the terms "**develop**", "**differentiate**" and "**mature**", as used to describe a stem cell, refer to the progression of a cell from the stage of having the potential to differentiate into at least two different cellular lineages to becoming a specialized and terminally differentiated cell. Such terms can be used interchangeably for the purposes of the present application.

The term "**expression**" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, in situ hybridization to cellular RNA or by PCR. Proteins encoded by a selected sequence can be quantified by various methods including, but not limited to, e.g., ELISA, Western blotting, radioimmunoassays, immunoprecipitation, assaying for the biological activity of the protein, or by immunostaining of the protein followed by FACS analysis.

"Expression control sequences" are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, internal ribosome entry sites (IRES) and the like, that provide for the expression of a coding sequence in a host cell. Exemplary expression control sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990).

The term "**feeder cell**" refers to a culture of cells that grows in vitro and secretes at least one factor into the culture medium, and that can be used to support the growth of another cell of interest in culture. As used herein, a "**feeder cell layer**" can be used interchangeably with the term "feeder cell." A feeder cell can comprise a monolayer, where the feeder cells cover the surface of the culture dish with a complete layer before growing on top of each other, or can comprise clusters of cells.

The term "**growth phase**" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are dividing at a constant rate. During this phase, cells are cultured for a period of time, and under such conditions that cell growth is maximized. The determination of the growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. "Period of time and under such conditions that cell growth is maximized" and the like, refer to those culture conditions that, for a particular cell line, are determined to be optimal for cell growth and division. During the growth phase, cells are cultured in nutrient medium containing the necessary additives usually at about 30-40 ° C., generally about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line, for instance a mammalian cell.

The term "**heterologous DNA**" refers to DNA which has been introduced into a cell that is derived from another source, or which is from the same source but is located in a different (i.e. non native) context. The term "**heterologous protein**" or "**recombinant protein**" refers to a protein encoded in all or part by heterologous DNA, or a protein that is expressed from expression control sequences (such as a promoter, or enhancer) created in whole or part by the heterologous DNA which activates the expression of an endogenous gene.

The term "**homology**" describes a mathematically based comparison of sequence similarities which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used.

The term "**homologous**" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (e.g., the immunoglobulin superfamily) in the same species of animal, as well as homologous proteins from different species of animal (for example, myosin light chain polypeptide, etc.; see Reeck et al., *Cell*, 50:667, 1987). Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term "**growth factor**" refers to Amphiregulin, Angiopoietin, Betacellulin, (Bone Morphogenic protein-13, Bone Morphogenic protein-14, Bone Morphogenic protein-2, Human BMP-3, Bone Morphogenic protein-4, Human BMP-5, Bone Morphogenic protein-6, Bone Morphogenic protein-7, Human CD135 Ligand / Flt-3 Ligand, Human Granulocyte Colony Stimulating Factor (G-CSF), Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Human

Macrophage Colony Stimulating Factor (M-CSF), Human Cripto-1, Human CTGF (Connective tissue growth factor), Human EGF (Epidermal Growth Factor), Human EG-VEGF (Endocrine-Gland-Derived Vascular Endothelial Growth Factor), Human Erythropoietin (EPO), Human FGF (Fibroblast Growth Factors 1-23), Human GDF-11, Human GDF-15, Human GDF-8, Human Growth Hormone Releasing Factor (GHRF, GRF, GHRH, Growth Hormone Releasing Hormone), Human Heparin Binding Epidermal Growth Factor (HB-EGF), Human Hepatocyte Growth Factor (HGF), Human Heregulin beta 1, Human insulin, Human IGF-1 (Insulin-like Growth Factor-1), Human IGF-2 (Insulin-like Growth Factor-2), Human IGFBP-1 (Insulin-like Growth Factor Binding Protein 1), Human IGFBP-3 (Insulin-like Growth Factor Binding Protein 3), intestinal trefoil factor (ITF), Human keratinocyte growth factors 1 & 2, Human Leukemia Inhibitory Factor (LIF), Human MSP, Human Myostatin, Human Myostatin, pro (propeptide), Human NRG1, Human NGF, Human Oncostatin M, Human Osteoblast Specific Factor 1 (OSF-1, Pleiotrophin), Human PD-ECGF (Platelet-derived endothelial cell growth factor), Human PDGF, Human PlGF, Human Placental Growth Factor 1 (PLGF1), Human Placental Growth Factor 2 (PLGF2), Human SCGF-a (Stem Cell Growth Factor-alpha), Human SCGF-b (Stem Cell Growth Factor-beta), Human Stem Cell Factor (SCF) / CD117 Ligand, Human Thrombopoietin (TPO, THPO), Human Transforming Growth Factor, Human TGF-alpha (Transforming Growth Factor-alpha, TGFa), Human TGF-beta 1 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 1.2 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 2 (Transforming Growth Factor-beta2, TGFb), Human TGF-beta 3 (Transforming Growth Factor-beta3, TGFb), Human VEGF (Vascular Endothelial Growth Factor), Human VEGF-121, Human VEGF-165, and Human VEGF-A

As used herein, "**identity**" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the

sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm can also be used to determine identity.

The terms "**immunoglobulin**" or "**antibody**" (used interchangeably herein) refers to a protein typically having a basic four-polypeptide chain structure consisting of two heavy and two light chains, the chains being stabilized, for example, by interchain disulfide bonds, which has the ability to specifically bind antigen. The term "single-chain immunoglobulin" or "single-chain antibody" (used interchangeably herein) refers to a protein having a two-polypeptide chain structure consisting of a heavy and a light chain, the chains being stabilized, for example, by interchain peptide linkers, which has the ability to specifically bind antigen. The term "domain" refers to a globular region of a heavy or light chain polypeptide comprising peptide loops (e.g., comprising 3 to 4 peptide loops) stabilized, for example, by beta-pleated sheet and/or intrachain disulfide bond. Domains are further referred to herein as "constant" or "variable", based on the relative lack of sequence variation within the domains of various class members in the case of a "constant" domain, or the significant variation within the domains of various class members in the case of a "variable" domain. Antibody or polypeptide "domains" are often referred to interchangeably in the art as antibody or polypeptide "regions". The "constant" domains of an antibody light chain are referred to interchangeably as "light chain constant regions", "light chain constant domains", "CL" regions or "CL" domains. The "constant" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "CH" regions or "CH" domains). The "variable" domains of an antibody light chain are referred to interchangeably as "light chain variable regions", "light chain variable domains", "VL" regions or "VL" domains). The "variable" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "VH" regions or "VH" domains). Immunoglobulins or antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer

amino acid residues than an intact or complete antibody or antibody chain. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Exemplary fragments include Fab, Fab', F(ab')2, Fabc and/or Fv fragments.

As used herein, the term "**increase**" or the related terms "**increased**", "**enhance**" or "**enhanced**" refers to a statistically significant increase. For the avoidance of doubt, the terms generally refer to at least a 10% increase in a given parameter, and can encompass at least a 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 95% increase, 97% increase, 99% or even a 100% increase over the control value.

The term "**isolated,**" when used to describe the cell culture components, i.e. albumin, or transferrin related proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

The term "**maturation-specific protein promoter**" refers to a promoter exhibiting substantially up-regulated activity (greater than 25%) during seed maturation.

The term "**mature plant**" refers to a fully differentiated plant.

"**Markers**" as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared

to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

Cells expressing "**markers of pancreatic endocrine lineage**" refer to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, NRx2.2, NRx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, and Pax6. Cells expressing markers characteristic of the pancreatic cell lineage include pancreatic β cells.

Cells expressing "**markers characteristic of endoderm lineage**" as used herein refer to cells expressing at least one of the following markers: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA-6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

Cells expressing pluripotency markers derived by the methods of the present invention express at least one of the following pluripotency markers selected from the group consisting of: ABCG2, cripto, FoxD3, Connexin43, Connexin45, Oct4, SOX-2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tral-60, and Tral-81.

Cells expressing "**markers characteristic of mesoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, GATA-6.

Cells expressing "**markers characteristics of ectoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: BMP-4, Noggin, Chordin, Otx2, Fox J3, Nestin, p63/TP73L, beta-III Tubulin.

"**Monocot seed components**" refers to carbohydrate, protein, and lipid components extractable from monocot seeds, typically mature monocot seeds.

As used herein, the terms "**native**" or "**wild-type**" relative to a given cell, protein, polypeptide, nucleic acid, trait or phenotype, refers to the form in which that is typically found in nature.

The terms "**operably linked**" and "**operatively linked**," as used interchangeably herein, refer to the positioning of two or more nucleotide sequences or sequence elements in a manner which permits them to function in their intended manner. In some embodiments, a nucleic acid molecule according to the invention includes one or more DNA elements capable of opening chromatin and/or maintaining chromatin in an open state operably linked to a nucleotide sequence encoding a recombinant protein. In other embodiments, a nucleic acid molecule may additionally include one or more nucleotide sequences chosen from: (a) a nucleotide sequence capable of increasing translation; (b) a nucleotide sequence capable of increasing secretion of the recombinant protein outside a cell; and (c) a nucleotide sequence capable of increasing the mRNA stability, where such nucleotide sequences are operatively linked to a nucleotide sequence encoding a recombinant protein. Generally, but not necessarily, the nucleotide sequences that are operably linked are contiguous and, where necessary, in reading frame. However, although an operably linked DNA element capable of opening chromatin and/or maintaining chromatin in an open state is generally located upstream of a nucleotide sequence encoding a recombinant protein; it is not necessarily contiguous with it. Operable linking of various nucleotide sequences is accomplished by recombinant methods well known in the art, e.g. using PCR methodology, by ligation at suitable restrictions sites or by annealing. Synthetic oligonucleotide linkers or adaptors can be used in accord with conventional practice if suitable restriction sites are not present.

A "**plant cell**" refers to any cell derived from a plant, including undifferentiated tissue (e.g., callus) as well as plant seeds, pollen, propagules, embryos, suspension cultures, meristematic regions, leaves, roots, shoots, gametophytes, sporophytes and microspores.

The terms "**polynucleotide**" and "**nucleic acid molecule**," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a

DNA polymerase with an appropriate primer. A nucleic acid molecule can take many different forms, e.g., a gene or gene fragment, one or more exons, one or more introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. As used herein, "DNA" or "nucleotide sequence" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

The term "**pluripotent stem cell**" encompasses stem cells obtained from embryos, fetuses or adult tissues. In one preferred embodiment, the pluripotent stem cell is an embryonic stem cell. In another embodiment the pluripotent stem cell is a fetal stem cell, such as a primordial germ cell. In another embodiment the pluripotent stem cell is an adult stem cell.

As used herein, the term "**pluripotent**" refers to a cell capable of at least developing into one of ectodermal, endodermal and mesodermal cells. As used herein the term "pluripotent" includes cells that are totipotent and multipotent. As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. The term "multipotent" refers to a cell that is not terminally differentiated.

A "**promoter**" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. As used herein, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. A transcription initiation site (conveniently defined by mapping with nuclease S1) can be found within a promoter sequence, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters can often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine- Dalgarno sequences in addition to the -10 and -35 consensus sequences.

A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources are well known in the art. Representative sources include for example, viral, mammalian, insect, plant, yeast, and bacterial cell types, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available on line or, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, the RSV promoter, the rice endosperm specific glutelin (Gt1) promoter, CaMV35S viral promoter. Inducible promoters include the Tet system, (US Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., Proc. Natl. Acad. Sci. (1996) 93 (8): 3346-3351; the T-RE$_x$™ system (Invitrogen Carlsbad, CA), LacSwitch® (Stratagene, (San Diego, CA) and the Cre-ER^T tamoxifen inducible recombinase system (Indra et al. Nuc. Acid. Res. (1999) 27 (22): 4324-4327; Nuc. Acid. Res. (2000) 28 (23): e99; US Patent No. 7,112,715; and Kramer & Fussenegger, Methods Mol. Biol. (2005) 308: 123-144) or any promoter known in the art suitable for expression in the desired cells.

The term "**protein of interest**" refers to any protein which may be useful for research, diagnostic or therapeutic purposes. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. Exemplary proteins of interest include, but are not limited to, molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; members of the TNF and TNF receptor (TNFR) family, like tumor necrosis factor-alpha and -beta, CD40 ligand, Apo-2 ligand/TRAIL, DR4, DR5, DcR1, DcR2, DcR3, OPG, Fas ligand; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse

gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TG-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; thrombopoietin (TPO); interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as. for example, a portion of the AIDS envelope, gp120; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and variants and/or fragments of any of the above-listed polypeptides: as well as antibodies against various protein antigens like CD proteins such as CD3, CD4, CD8, CD19. CD20 and CD34; members of the ErbB receptor family such as the EGF receptor. HER2, HER3 or HER4 receptor: cell adhesion molecules such as LFA-1, Mac1, p150.95. VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C; an Apo-2L receptor such as Apo-2 (DR5), DR4, DcR1, DcR2, DcR3; and variants and/or fragments of the above-identified antibodies etc. In one embodiment of the invention, a protein of interest will comprise a protein which itself is capable of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

The term "**production phase**" of the cell culture refers to the period of time during which cell growth has reached a plateau. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The term "**produced in a plant**" when used to describe the cell culture components disclosed herein, means that the protein has been obtained directly or indirectly from a plant cell. With respect to recombinant proteins, the term "produced in a plant" means that the recombinant protein has been expressed within a plant cell, and then purified from host cells proteins and lipids. Proteins that have been produced in a plant may be differentiated from proteins that have purified from other organisms by at least three criteria. 1) Proteins produced in a plant will typically comprise different patterns of post-translational modifications compared to proteins produced in different organisms, which may be readily identified by mass spectrum analysis. 2) Proteins produced in a plant will typically comprise a different set of bound co-factors, lipids and contaminating proteins compared to the same protein produced in a different expression system, or purified from a different source. 3) Proteins produced in a plant may exhibit a different biological activity compared to the same protein produced or purified from a different source. The difference in activity in the protein produced in a plant may arise from a difference in the conformation, folding, aggregation state, post-translational modifications, or bound factors or co-purified contaminating proteins of the protein produced in a plant compared to a protein produced or isolated from a different organism. Differences in the specific activity of a protein produced in a plant may be readily established by routine assays using defined concentrations and assay conditions, for example, using cell growth, and factor synergism assays, as disclosed in Examples 11 to 19. Specifically a protein produced in a plant may exhibit at least at least 10 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. Preferably, a protein produced in a plant may exhibit at least at least 20 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 30 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 40 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. Even more preferably, a protein produced in a plant may exhibit at least at least 50 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein.

The terms "**isolated**" or "**purified**" when used to describe the cell culture components disclosed herein, means protein that has been identified and separated and/or recovered from a

component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

The term "**recombinant protein**" or "**recombinant polypeptide**" refers to an exogenous, i.e., heterologous or foreign polypeptide, to the cells producing the polypeptide.

The term "**stress**" in the context of apoptosis or cell culture refers to non-optimal conditions for tissue culture including any combination of the following; the presence of toxins, nutrient or growth factor depletion or withdrawal, hypoxia, thermal stress (temperature is too high or too low compared to the preferred range), loss of cell-cell contacts, viral infection, osmotic stress (osmolality is too high or too low compared to the preferred range), oxidative stress, cell density (cell density is too high or too low compared to the preferred range), and pH stress (pH is too high or too low compared to the preferred range).

The term "**transformation**" refers to the transfer of one or more nucleic acid molecules into a host cell or organism. Methods of introducing nucleic acid molecules into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, scrape loading, ballistic introduction or infection with viruses or other infectious agents. "**Transformed**", "**transduced**", "**transgenic**", and "**recombinant**" refer to a host cell or organism into which a recombinant or heterologous nucleic acid molecule (e.g., one or more DNA constructs or RNA, or siRNA counterparts) has been introduced. The nucleic acid molecule can be stably expressed (i.e. maintained in a functional form in the cell for longer than about three months) or non-stably maintained in a functional form in the cell for less than three months i.e. is transiently expressed. For example, "transformed," "transformant," and "transgenic" cells have been through the transformation process and contain foreign nucleic acid. The term "untransformed" refers to cells that have not been through the transformation process.

The term "**transition phase**" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as pH, ion concentration, and temperature may shift from growth conditions to production conditions.

The term "**seed product**" includes, but is not limited to, seed fractions such as de-hulled whole seed, flour (seed that has been de-hulled by milling and ground into a powder) a seed extract, preferably a protein extract (where the protein fraction of the flour has been separated from the carbohydrate fraction), malt (including malt extract or malt syrup) and/or a purified protein fraction derived from the transgenic grain.

"**Seed maturation**" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

The term "**sequence similarity**" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (see Reeck et al., supra). However, in common usage and in the instant application, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 85%, and more preferably at least about 90% or at least about 95% of the nucleotides match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm known such as BLAST, FASTA, DNA Strider, CLUSTAL, etc. An example of such a sequence is an allelic or species variant of the specific genes of the present invention. Sequences that are substantially homologous may also be identified by hybridization, e.g., in a Southern hybridization experiment under, e.g., stringent conditions as defined for that particular system.

Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acid

residues are identical, or when greater than about 90% of the amino acid residues are similar (i.e., are functionally identical). Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above. The program may use the local homology algorithm of Smith and Waterman with the default values: Gap creation penalty = -(1+1/*k*), *k* being the gap extension number, Average match = 1, Average mismatch = -0.333.

As used herein and in the appended claims, the singular forms "a," "an," and "the," include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a molecule" includes one or more of such molecules, "a reagent" includes one or more of such different reagents, reference to "an antibody" includes one or more of such different antibodies, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993): *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds. (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands;, DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, In Situ Hybridization: Principles and Practice; Oxford University Press; M. J. Gait (Editor), 1984, Oligonucleotide Synthesis: A Practical Approach, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press; Using Antibodies: A Laboratory Manual: Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies: A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-3,4-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, N.Y., Marcel Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other

Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory. ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods, compositions, reagents, cells, similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described herein. All publications mentioned herein are incorporated herein, including all figures, graphs, equations, illustrations, and drawings, to describe and disclose specific information for which the reference was cited in connection with.

I. Methods of using the supplements of the invention

Frequently, cell culture media is supplemented with serum at concentration of 5 to 10%. Serum is a complex mixture containing undefined essential matter for cell growth. Commonly used serum is fetal calf serum (FCS) and fetal bovine serum (FBS). Due to its complex nature, lack of consistency, and the potential risk for pathogen contamination, cell culture industries and regulatory agencies are seeking alternatives to adding serum to cell culture media.

Cell culture components and cell culture supplements added to basal media serve distinct functions in promoting cell growth. These protein components are typically animal-derived. With the increase in the concern of potential pathogen contamination from animal sourced material, cell culture industries are trying to develop animal-component-free media. While some successes have been made with the use of plant-based hydrolysates and recombinant proteins produced using microbial systems, plant-based hydrolysates are an undefined mixture of components and recombinant protein from microbial sources are too expensive to use in routine cell culture. An alternative source of non-animal-derived components would be beneficial for industries that depend on cell culture techniques.

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

According to further embodiments, the combination of proteins that is used in the methods of culturing cells and supplements of the invention provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, the supplements comprising one combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

In another aspect, the supplements comprises a combination of transferrin related proteins and albumin which provide a synergistic effect on cell growth and productivity.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, adding a supplement of the present invention to the cell culture medium.

The claimed supplements are useful in a wide range of applications for tissue and cell culture and recombinant protein production where they provide for significant improvements in preventing apoptosis and improving cell viability during tissue culture, and in particular in response to stress.

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. These changes include, changes to the cell membrane such as loss of membrane asymmetry and attachment, cellular blebbing cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, cytokines, which may be present to different degrees in tissue culture media. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis, and thus influence overall cell viability. A number of intracellular components, including ATP content, calcium level, and a number of apoptotic and anti-apoptotic genes also help regulate apoptosis. A cell may initiate intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. Stress inducing agents encountered during tissue culture include for example toxins, associated with tissue culture components such as endotoxins, and heavy metals that leach from plastic ware, transfection reagents (e.g. Lipofectamine and similar lipid based transfection reagents), viral transformation, nutrient and growth factor deprivation, associated with serum free culture, or cell differentiation protocols hypoxia and oxidative stress associated with high density culture in a bioreactor and increased intracellular calcium concentration, for example, by damage to the membrane caused by detergents and electroporation.

Before the actual process of cell death occurs, the apoptotic signals must overcome regulatory proteins which act as gatekeepers overseeing the activation of the apoptosis pathway. In vivo, this step allows the process to be stopped, should the cell no longer need to die. Several proteins are involved at this step, though two main mechanisms of regulation have been identified and include those associated with mitochondria functionality, and those directly involved in transducing the signal via adaptor proteins to the apoptotic mechanisms.

Cells grown under cell culture conditions may experience cellular stresses associated with routine tissue culture procedures, as described above which may trigger apoptotic signals and increase the susceptibility of the cells to apoptosis. For example, nutrient deprivation associated with serum free culture, oxidative stress associated with high density growth in a bioreactor, the use of cytotoxic compounds associated with DNA transfection reagents, and thermal stresses associated with cryopreservation, may predispose the cell to enter apoptosis. By enhancing the ability of a cell to survive such signals it is possible to improve cell viability during these procedures, by preventing the cells commitment to cell death, thereby improving the success and utility of these approaches.

Recently a number of genes in eukaryotic cells have been identified which inhibit the onset or reduce the effects of apoptosis. Some of these genes inhibit caspase dependent apoptotic pathways in the cell, and in fact transfecting cells with anti-apoptotic genes may be useful in prolonging the life and productivity of transfected cells grown under biologically demanding conditions. (US Patent Nos., 6,586,206, 7,531,327; US Patent Application US 2009/0170165; US2009/0181426).

Additionally the addition of exogenous heat shock proteins has in some cases been shown to improve the survival of cells in culture under a variety of conditions. (Novoselova et al., J. Neurochem. 94 597-606 (2005); Tidwell et al., Cell Stress & Chap 9 (1) 88-90 (2004); Guzhova et al., Cell Stress & Chap. 3 (1) 67-77 (1998); Hounenou et al., Cell Stress & Chap 1 (3) 161-166 (1996); Johnson et al., In vitro Cell. Dev. Biol., 29A 807-812 (1993).

The present invention is based in part on the demonstration that plant derived recombinant cell culture component proteins surprisingly enhanced the cell growth and viability when added in specific mixtures to mammalian cells grown in culture. Surprisingly some combinations of proteins provide for a synergistic effect on cell growth and productivity which provides unexpected benefits compared to the use of the individual proteins. Specifically, such supplements comprising plant derived recombinant albumin and transferrin related proteins result in improved culture viability, extended cell survival, improved rates of cell growth and improved yields of recombinant proteins produced from tissue culture bioreactors. Because the supplements show unexpectedly improved activity and stability they offer significant improvements compared to the use of standard recombinant or purified proteins.

The supplements disclosed in the present application are useful, for example, for improving cell viability and in accelerating the rate of cell growth of cells grown in culture. In one aspect, the supplements of the invention are useful for improving or enhancing the yield of the recombinant proteins from the cell cultures. Further improvements provided by the invention are described in detail below.

In one embodiment, the present invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media.

In one embodiment, the present invention includes a method for reducing the accumulation of lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method of reducing time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor.

In one embodiment, the present invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium.

In one embodiment, the present invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection.

In one embodiment, the present invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing.

In one embodiment, the present invention includes a method for improving the rate of cell growth or viability of stem cells grown in culture comprising the addition of a supplement of the present invention to the cell culture media.

In one embodiment, the present invention includes a method for improving the yield of a recombinant product produced from cells in culture comprising the addition of a supplement of the present invention to the cell culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the purification of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for reducing the proteolysis of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the bioactivity of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the stability of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the assembly of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a more human pattern of glycosylation of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a recombinant product produced from cells in culture with less immunogenicity, comprising the addition of a supplement comprising recombinant albumin to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In any of these methods, the supplements of the invention, by increasing host cell viability in culture (and during fermentation), provide for a simple and cost effective method to increase the yield, and or purity, bioactivity, stability and assembly of functional recombinant protein. Additionally, the supplements of the invention, by decreasing or inhibiting apoptosis in the cell culture, can decrease the number or presence of adverse proteases in the culture media and protect the expressed protein of interest against proteolytic degradation, thereby increasing the quality of the protein of interest produced, as evidenced by increased amounts of active protein, and increased yields of intact protein. Additionally Applicants have found that the supplements

of the invention may protect the cells against potential adverse effects of agents like detergents, heavy metals and endotoxin contaminates present in the culture components, or protect the cells from toxic reagents introduced to the cells during transfection or cryopreservation.

In any of the claimed methods, the supplements of the invention can be added directly, or admixed, to the culture media at any convenient time, for example when changing the media, passaging the cells, or when plating out the cells at low density. Optionally, the supplement is added to the culture media at the beginning (at the time of initiating, day 0) of the cell culturing process. In one aspect the supplements of the invention may be added before an anticipated stressful event, for example before cryopreservation, transfection or serum withdrawal, etc.

In another aspect, the supplement is added to the culture media during the culturing of the cells prior to the point when induction of typically apoptosis occurs. For example, during a large scale cell culture, induction of apoptosis can be observed on about day 3 or day 4 of the culture, and therefore, the supplement will preferably be added prior to day 3 or day 4. Optionally, a desired quantity of the supplement is added throughout, or for the duration of, the cell culture, for instance, on a daily basis for the entire fermentation. As an example, for a 5 day culture, the supplement could be added at day 0, and every 24 hours thereafter until the culture is terminated.

Accordingly in one embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a bioreactor by adding a supplement of the invention to the bioreactor. In one embodiment, the bioreactor comprises bacterial cells. In another aspect the bioreactor comprises yeast cells. In another aspect the bioreactor comprises plant cells. In another aspect the bioreactor comprises mammalian cells.

In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in bacterial cells, by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in yeast cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a plant cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in insect cells by

adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in mammalian cells by adding the supplement of the invention to the cell culture.

In another embodiment, the invention provides a method to increase the yield of the production phase of a cell culture system and thereby increase the productivity of a bioreactor by adding the supplement of the invention to the cell culture system prior to, or during the production phase of the cell culture system.

In one aspect of this method the yield of the production phase is increased by about 10%. In one aspect of this method the yield of the production phase is increased by about 20%. In one aspect of this method the yield of the production phase is increased by about 30%. In one aspect of this method the yield of the production phase is increased by about 40%. In one aspect of this method the yield of the production phase is increased by about 50%. In one aspect of this method the yield of the production phase is increased by about 60%. In one aspect of this method the yield of the production phase is increased by about 70%. In one aspect of this method the yield of the production phase is increased by about 80%. In one aspect of this method the yield of the production phase is increased by about 90%. In one aspect of this method the yield of the production phase is increased by about 100%. In one aspect of this method the yield of the production phase is increased by about 200%. In one aspect of this method the yield of the production phase is increased by about 500%.

In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which the supplement was not added. In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which is added a supplement lacking either a transferrin related protein, or recombinant serum albumin. In one aspect, the yield is increased compared to the summed effect of the recombinant albumin and the transferrin related protein when added alone when measured under the same culture conditions.

In another embodiment, the invention provides a method to produce a protein of interest at a temperature that is elevated compared to normal growth conditions for the production of that protein, comprising the addition of a supplement of the invention to cells expressing the protein of interest.

In another embodiment, the invention provides a method to decrease the amount of aggregates formed in a cell culture expression system by aggregate prone proteins of interest comprising the addition of a supplement of the invention to the cell culture expression system, whereby the aggregation state of the protein is reduced.

In another embodiment, the invention provides a method to increase the activity of a protein of interest protein expressed by a cell by preventing the denaturation and aggregation of the recombinant protein comprising the addition of a supplement of the invention to the cell, whereby the specific activity of the protein of interest is increased.

In another embodiment, the invention provides a method to improve the expression of proteins in a cell culture expression system that are aggregation prone, cause precipitation to occur, or are toxic themselves to the cells comprising the addition of a supplement of the invention to the cell culture expression system, whereby the expression of the protein of interest is increased.

The amount of supplement to add in any of these methods will depend on various factors, for instance, the type of host cell, the cell density, protein of interest and culture conditions, etc. Determining the desired concentration of supplement to be added to the culture media is within the skill in the art and can be ascertained empirically by routine optimization and without undue experimentation.

The skilled artisan will readily appreciate that different cell types will have different magnitudes of responses to the supplement of the invention, and this will be determined, to some degree, by the amount or type of the proteins in the supplement. Additionally different densities of cells will require appropriate adjustment in the total amount of supplement as well as the concentration of the individual proteins added to the culture to account for the increased cell number. Additionally cells grown in suspension culture or via adherent culture will have different membrane surface areas available for protein entry and will typically exhibit different rates and degrees of response. Therefore, one should choose a concentration which provides for a sufficient inhibition of apoptosis, or increase in viability, or net cell growth. Typically the supplements of the invention will be added to a final concentration of about 0.1 %, about 0.5 %, about 1%, about 2%, about 3 %, about 4 %, about 5 %, about 6%, about 7%, about 8%, about 9%, about 10 %, about 11 %, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 25%, about 30%, about 40%, or about 50% wt/wt, or wt /volume.

There will typically be an upper range of concentration of the supplement beyond which further increases in cell survival do not occur. As described in the Examples below, Applicants have found that the supplements of the invention can inhibit apoptosis when added to cell cultures at a concentration of about 200 mg /L to about 2g/L, or more preferably about 200 mg/L to about 1000 mg/L, or more preferable about 250 to about 500 mg/L.

II. Supplements

The cell culture media, methods for producing supplemented cell culture media, and methods of culturing cells using fortified cell culture media in accordance with the present invention may include components derived from animal, plant, bacterial, yeast, and insect sources. According to one aspect of the invention, the components are derived from plant sources, and preferably from monocot plants. The components may be combined with inorganic salts such as NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, and $MgCl_2$ and other ingredients such as amino acids, vitamins, growth factors, sugars, and antibiotics to form a variety of different cell culture media.

According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to another aspect, one of the proteins is albumin, and at least one additional protein is selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof.

According to further embodiments, the combination of proteins that is used in the methods of producing the supplemented cell culture media provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further

embodiments, one combination of proteins that may be used in the methods of producing the cell culture media includes a combination of albumin with at least one other protein selected from the group consisting of lactoferrin, transferrin, melanotransferrin, and ovotransferin.

In one aspect the supplements of the invention comprise plant derived recombinant human serum albumin.

The term "**albumin**", refers to all naturally-occurring and synthetic forms of albumin. Preferably, the term "albumin" refers to recombinant albumin. In one aspect the albumin is from a vertebrate. In one aspect the albumin is from a mammal. In a further embodiment the albumin is human. In another aspect, the recombinant albumin is produced from a plant cell. In one particularly preferred embodiment the recombinant albumin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of albumin are listed below in **Table D 1.**

Table D1

Exemplary Albumin genes

Species	Gene Bank Accession number
Human	NP_000468.1
Pan troglodytes	XP_517233.2
Canis lupus familiaris	XP_855557.1
Bos taurus	NP_851335.1
Mus musculus	NP_033784.1
Rattus norvegicus	NP_599153.1
Gallus gallus	NP_990592.1

It will be understood that for the recombinant production of albumin in different species it will typically be necessary to codon optimize the nucleic acid sequence of the gene for the host organism in question. Such codon optimization can be completed by standard analysis of the preferred codon usage for the host organism in question, and the synthesis of an optimized nucleic acid via standard DNA synthesis. A number of companies provide such services on a fee for services basis and include for example, DNA2.0. (CA, USA) and Operon Technologies. (CA, USA).

The albumin may be in its native form, i.e., as different allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the albumin, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the albumin.

Fragments of native or synthetic albumin sequences may also have the desirable functional properties of the peptide from which they derived and may be used in any of the methods of the invention. The term "fragment" as used herein thus includes fragments of albumin provided that the fragment retains the biological or therapeutically beneficial activity of the whole molecule.

For example, albumin contains at least 2 high affinity multi- metal binding sites for a number of physiologically important metals ions including copper, zinc, cadmium and nickel. (Carter et al., Advances in Protein Chemistry 45 153-203 (1994): Bai et al., J. Inorg Biochem 70 (1) 33-39 (1998), Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009); US Patent No. 6,787,636). Since trace amounts of these metals are typically present in the recombinant production of albumin, a significant amount of these metal ions can be become chelated to the protein. The binding of these ions, and in particular the binding of cadmium and nickel to recombinant albumin is associated with cellular toxicity of the protein when added to cells as a tissue culture component.

Accordingly, in one aspect, the albumin of the present invention can comprise a fragment of albumin that includes the deletion of one or amino acids involved in the multi-metal binding sites of albumin. In one aspect the albumin fragment is created by the deletion of one or more amino acids at the N-terminus of the mature protein. In another aspect the albumin can comprise one or more deletions or mutations of any of the amino acids involved in the N-terminal metal binding site of albumin. In one aspect, the amino acids to be deleted or mutated are independently selected from the first 10 amino acids of the mature protein.

The term "derivative" as used herein thus refers to albumin sequences or fragments thereof, which have modifications as compared to the native sequence. Such modifications may be one or more amino acid deletions, additions, insertions and/or substitutions. These may be contiguous or non-contiguous. Representative variants may include those having 1 to 20, or more preferably 1 to 15, 1 to 10, or 1 to 5 amino acid substitutions, insertions, and / or deletions as compared to any of genes listed in Tables D1. The substituted amino acid may be any amino acid, particularly one of the well-known 20 conventional amino acids (Ala (A); Cys (C); Asp (D); Glu (E); Phe (F); Gly (G); His (H); Ile (I); Lys (K); Leu (L); Met (M); Asn (N); Pro (P); Gln (Q); Arg (R); Ser (S); Thr (T); Val (V); Trp (W); and Tyr (Y)). Any such variant or derivative of albumin may be used in any of the methods of the invention.

Accordingly, the albumin of the invention can comprise amino acid deletions, insertions or mutations in any of the functional binding domains of albumin. In one aspect the albumin may comprise a mutation in a binding domain of albumin. In one aspect the mutated binding domain is a domain involved in the binding of aspirin, warfarin, diazepam, digitoxin, dlofibrate, ibuprofen or AZT, as outlined is US Patent No. 5,780,593, or a multimetal binding site as outlined in Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009).

Thus, the albumin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native albumin amino acid sequences, for example, to any of the native albumin gene sequences listed in **Table D1**. Alternatively, the albumin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with albumin listed in **Table D1**. In a preferred embodiment, the albumin for use in any of the methods of the present invention is at least 80% identical to the mature secreted human serum albumin (**SEQ. ID. No. 1**) as shown underlined in the below (Swiss-Prot P02768):

MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF

EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP

ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF

FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV

ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK

ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR

RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE

QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV

LRQLCVLHEK LPVSLRVLKC CLESLVDKRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV AASQAALGL

Fusion proteins of albumin to other proteins are also included, and these fusion proteins may enhance, activity, targeting, stability or potency.

Chemical modifications of the native albumin structure which retain or stabilize albumin activity or biological half-life may also be used with any of the methods described herein. Such chemical modification strategies include without limitation pegylation, glycosylation, and acylation (see Clark et al.: *J. Biol. Chem.* 271(36): 21969-21977, 1996; Roberts et al.: *Adv. Drug. Deliv. Rev.* 54(4): 459-476, (2002); Felix et al.: *Int. J. Pept. Protein. Res.* 46(3-4): 253-264, (1995); Garber Diabetes Obes. Metab. 7 (6) 666-74 (2005)) C- and N-terminal protecting groups and peptomimetic units may also be included.

Isomers of the native L-amino acids, e.g., D-amino acids may be incorporated in any of the above forms of albumin, and used in any of the methods of the invention. All such variants, derivatives, fusion proteins, or fragments of albumin are included, may be used in any of the methods claims or disclosed herein, and are subsumed under the term "albumin".

In one aspect of the supplements of the invention the albumin is recombinant human serum albumin that has been produced in a plant. In another aspect, the albumin comprises less than about 2% aggregated albumin. In another aspect the albumin comprises less than about 1 % aggregated albumin.

In one embodiment, the supplements of the invention comprise preparations of the co-purified recombinant albumin and rice hsps that are also essentially free of detergents and endotoxins which would otherwise mask or inhibit the positive impact of the hsp. In one aspect the supplements of the invention have less than about 1 EU of endotoxin, and the albumin is at least about 95 % pure. In another aspect the supplements of the invention comprise a composition comprising recombinant albumin bound to a heat shock protein. In another aspect the supplements of the invention comprise recombinant albumin and a rice hsp70 homolog. In one aspect the rice hsp70 homolog is selected from HSP70, Bip and rice stromal protein.

The terms "**heat shock protein**", "**HSP**" or "**hsp**", as used herein includes all naturally-occurring and synthetic forms of the heat shock protein super family that retain anti-apoptotic activity. Such heat shock proteins include the small heat shock proteins/HSPB family, Hsp40/DnaJ family, HSP70/ HSPA family, HSP90 / HSPC family, HSP110/ HSPH family and chapererone family, as well as peptide fragments and protein complexes of two or more heat shock proteins or nucleotide exchange factors (for example, complexes of HSP70 & HSP40) derived therefrom. The heat shock proteins may be in their native form, i.e., as different variants as they appear in nature in different species which may be viewed as functionally equivalent variants, or they may be functionally equivalent natural derivatives thereof, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical derivatives, including post-translational modifications and degradation products of the HSPs, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, oxidatized, isomerized, and deaminated variants of the HSP.

The term "**transferrin related protein**" refers to transferrin family proteins including transferrin, lactoferrin, melanotransferrin, and ovotransferin. The crystal structures of transferrin family members across all species that have been solved to date show a high degree of structural similarity, which is not surprising since transferrin, lactoferrin and ovotransferrin share 60-80% sequence identity. (Wally and Buchanan, Biometals (2007) 20 249-262). All transferrin related proteins contain two domains of around 340 residues, which are thought to have evolved from an ancient duplication event. For serum transferrin, ovotransferrin and lactoferrin each of the duplicated lobes binds one atom of Fe (III) and one carbonate anion. With a few notable exceptions each iron atom is coordinated to four conserved amino acid residues: an aspartic acid, two tyrosines, and a histidine, while anion binding is associated with an arginine and a threonine in close proximity. (Anderson et al., (1989) J. Mol. Biol. 209 711-734).

Human transferrin and lactoferrin share over 61 % sequence identity and have similar three dimensional structures. Transferrins transport iron in the blood by picking up free ferric iron and delivering it to cells in a receptor-mediated endocytotic process in which the TF-receptor complex is internalized, iron is released in the endosome, and the complex is recycled to the cell surface where the TF is released. In contrast, no iron transport role for lactoferrin or ovotransferrin is known. By comparison, these proteins are thought to function to prevent invading bacteria from acquiring iron, by sequestering all available iron. Human transferrin

releases iron at a higher pH than does human lactoferrin which likely relates to the need for human transferrin to release iron after endocytosis into the endosome, and the need for lactoferrin to hold onto iron in low pH environments such as the stomach (Baker & Backer (2004) Biometals 17 209-216).

The term "**transferrin**" refers to all naturally-occurring and synthetic forms of transferrin. In one aspect, the term "transferrin" refers to recombinant transferrin. In one aspect, the term "transferrin" refers to plasma derived transferrin. In one aspect the transferrin is from a vertebrate. In one aspect the transferrin is from a mammal. In a further embodiment the transferrin is human. In another aspect the recombinant transferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant transferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of transferrin are listed below in **Table D2**.

Table D2

Exemplary Transferrin genes

Species	Gene Bank Accession number
Homo sapiens	NP_001054.1
Canis lupus familiaris	XP_864550.1
Bos taurus	NP_803450.2
Mus musculus	NP_598738.1
Rattus norvegicus	NP_001013128.1
Gallus gallus	NP_990635.1
Danio rerio	NP_001015057.1

The transferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the transferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the transferrin.

The transferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native transferrin amino acid sequences, for example, to any of the native transferrin gene sequences listed in **Table D2**. Alternatively, the transferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with transferrin listed in **Table D2**. In a preferred embodiment, the transferrin for use in any of the methods of the present invention is at least 80% identical to the mature human transferrin.

The term "**Lactoferrin**" refers to all naturally-occurring and synthetic forms of Lactoferrin. In one aspect, the term "Lactoferrin" refers to recombinant Lactoferrin. In one aspect, the term "Lactoferrin" refers to milk derived Lactoferrin. In one aspect the Lactoferrin is from a vertebrate. In one aspect the Lactoferrin is from a mammal. In a further embodiment the Lactoferrin is human. In another aspect the recombinant Lactoferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant Lactoferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of Lactoferrin are listed below in **Table D3**

Table D3 Exemplary Lactoferrin genes	
Species	Gene Bank Accession number
Homo sapiens	AAA59511.1
Sus scrofa	AAA31059.1
Camelus dromedarius	CAB53387.1
Bos taurus	AAA30610.1
Equus caballus	CAA09407.1

The Lactoferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of Lactoferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of Lactoferrin. Lactoferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native Lactoferrin amino acid sequences, for example, to any of the native Lactoferrin gene sequences listed in **Table D3**. Alternatively, the Lactoferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with Lactoferrin listed in **Table D3**. In a preferred embodiment, the Lactoferrin for use in any of the methods of the present invention is at least 80% identical to the mature human Lactoferrin.

The term "**melanotransferrin**" refers to all naturally-occurring and synthetic forms of melanotransferrin. In one aspect, the term "melanotransferrin" refers to recombinant melanotransferrin. In one aspect, the term "melanotransferrin" refers to melanotransferrin purified from a cell or tissue. In one aspect the melanotransferrin is from a vertebrate. In one aspect the melanotransferrin is from a mammal. In a further embodiment the melanotransferrin is human. In another aspect the recombinant melanotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant melanotransferrin is produced from

transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of melanotransferrin are listed below in **Table D4**

Table D4 Exemplary **melanotransferrin** genes	
Species	Gene Bank Accession number
Homo sapiens	AAA59992.1
Oryctolagus cuniculus	NP_001075461.1
Mus musculus	NP_038928.1
Canis lupus familiaris	XP_545158.2
Gallus gallus	CAA63003.1

The term "ovotransferrin" refers to all naturally-occurring and synthetic forms of ovotransferin. In one aspect, the term "ovotransferrin" refers to recombinant ovotransferrin. In one aspect the ovotransferin is from a vertebrate. In one aspect the ovotransferin is from an avian. In another aspect the recombinant ovotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant ovotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of ovotransferrin include CAA26040.1.

The term "**insulin related growth factor" or "IGF-1"** refers to all naturally-occurring and synthetic forms of insulin related growth factor, including IGF-1, IGF-1A, IGF-1B, IGF-2A, and IGF-2B. In one aspect, the term "insulin related growth factor" refers to recombinant insulin related growth factor. In one aspect, the term "insulin related growth factor" refers to insulin related growth factor purified from a cell or tissue. In one aspect the insulin related growth factor is from a vertebrate. In one aspect the insulin related growth factor is from a mammal. In a further embodiment the insulin related growth factor is human. In another aspect the recombinant insulin related growth factor is produced from a plant cell. In one particularly preferred embodiment the recombinant insulin related growth factor is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of insulin related growth factor are listed below in **Table D5**

Table D5 Exemplary insulin related growth factor genes	
Species	Gene Bank Accession number
Homo sapiens	P01343 P05019 CAA01954.1 CAA40093.1 CAA40092.1
Bos taurus	NP_001071296
Mus musculus	NP_001104746.1 NP_034642.2 NP_908941.1 NP_001104745.1 NP_001104744.1
Xenopus laevis (African clawed frog)	Q90WW4
Danio rerio (zebrafish	NP_571900

The **insulin related growth factor** may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of **insulin related growth factor**, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of **insulin related growth factor**. **Insulin related growth factor** which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native **insulin related growth factor** amino acid sequences, for example, to any

of the native **insulin related growth factor** gene sequences listed in **Table D5**. Alternatively, the **insulin related growth factor** may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with **insulin related growth factor** listed in **Table D5**. In a preferred embodiment, the **insulin related growth factor** for use in any of the methods of the present invention is at least 80% identical to the mature human **insulin related growth factor**.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 5, about 1 to 10, about 1 to 15, about 1 to 20 or about 1 to 25. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 0.5 g/L, In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components,

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 1 about 1 to 2, about 1 to 3, about 1 to 4 or about 1 to 5, In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.5 to 0.8 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components,

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.5 about 1 to 0.75, about 1 to 1.0 about 1 to 1.25, about 1 to 1.5, about 1 to 1.75 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.8 to 1.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components,

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.25 about 1 to 0.33, about 1 to 0.66 about 1 to 1.00, about 1 to 1.33, about 1 to 1.66 or about 1 to 2.0. In one aspect of any of these

supplements, the concentration of lactoferrin in the culture is about 1.0 to about 1.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.8, about 1 to 1 or about 1 to 1.2. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 1.5 g/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 20, about 1 to 100, or about 1 to 200. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 500, about 1 to 1000, or about 1 to 2000. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 3000, about 1 to 4000, or about 1 to 5000. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to

the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and albumin mixed in a ratio of transferrin to albumin of about 1 to 80, about 1 to 100 or about 1 to 120. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 3 to about 8 u g/L. In one aspect the supplement exhibits at least an 80% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of IGF-1 and recombinant albumin mixed in a ratio of IGF-1 to albumin of about 1 to 100000, about 1 to 10000, or about 1 to 1000. In one aspect of any of these supplements, the concentration of IGF-1 in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In another aspect of the invention, the supplements contain one or more additional factors selected from the group consisting of a growth factor, sodium selenate and ethanolamine

In another aspect of the invention, the growth factors are independently selected from insulin, Epidermal Growth Factor (EGF), Fibroblast Growth Factors 1-23 (FGF), Insulin-like Growth Factor-1(IGF), keratinocyte growth factors 1 & 2(KGF), and Leukemia Inhibitory Factor (LIF). In one aspect the growth factor is insulin. In another aspect the growth factor is transferrin.

In one aspect, the supplements of the invention may be prepared by mixing the isolated cell culture components in aqueous solution.

Liquids of known concentration can also be combined containing one component part A (albumin or another cell culture component), to a liquid containing part B (such as a transferrin related protein or IGF-1) to obtain a ratio that contains the desired ratios of the components Powdered, lyophilized, or otherwise dried powder (albumin) can be added directly to an

aqueous solution containing the cell culture component (such as transferrin related protein) in order to obtain a ratio based on dry weight of albumin at any desired range with respect to the other cell culture components. Powdered, lyophilized, or otherwise dried albumin can also be blended with the cell culture component powder on a mass to mass basis to obtain a ratio that is completely based on gravimetrics. The resulting powder can be dissolved at concentrations ranging from very low (picomolar) to very high concentrations (millimolar) in suitable buffers that are common to the art to reconstitute the cell culture components.

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin and one or more transferrin related proteins and /or IGF-1. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of transferrin related protein in the composition may vary from 50% to about 0.0001% of weight of the cell culture component. In other aspects the amount of transferrin related protein in the composition may vary from about 0.01% to about 0.02%, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08%. In another aspect the amount of transferrin related protein in the composition is greater than about 0.01%, or more preferably greater than about 0.05%, or more preferably greater than about 0.1% wt /wt, or more preferably greater than about 0.2% wt/wt transferrin related protein with respect to albumin.

In one aspect of any of the claimed supplements, the recombinant albumin is essentially free of endotoxin and detergents. In another aspect the albumin has less than about 1 EU /mg of endotoxin. In yet another aspect, the albumin contains less than about 10 ppm detergent. In another aspect of any of the claimed supplements, the albumin has a purity of greater than 95 %.

In another of any of the claimed supplements, the supplement comprises recombinant albumin which is bound to a rice heat shock protein, wherein the complex has less than about 1 EU of endotoxin and is at least 95 % pure with respect to the albumin. In one aspect the recombinant albumin is produced in rice.

In another aspect of any of these methods the supplement contains recombinant albumin as the cell culture component, and the albumin is essentially free of aggregated albumin. In another

aspect of any of these supplements the recombinant albumin has less than about 2 % aggregated albumin.

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin with one or more bound hsps. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of hsp in the albumin composition may vary from 5 % to about 0.001% of weight of albumin. In other aspects the amount of hsp in the albumin composition may vary from about 0.01% to about 0.02 %, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08 %. In another aspect the amount of hsp in the albumin composition is greater than about 0.02%, or more preferably greater than about 0.03%, or more preferably greater than about 0.04% wt /wt, or more preferably greater than about 0.05% wt/wt hsp with respect to albumin.

III. Production of Cell culture media components

Albumin and transferrin related proteins, as well as other cell culture media components for use in the supplements of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (see below), or by chemical synthesis, using, for instance, automated peptide synthesizers, or any combination of such methods. The means for preparing such polypeptides are well understood in the art.

For recombinant production, host cells can be genetically engineered to incorporate nucleic acids encoding the cell culture media component. Typically the nucleic acid will be codon optimized for high level expression in the expression system of choice, and incorporated into an expression vector to enable the expression of the protein of interest in the host cell. Vectors can exist as circular, double stranded DNA, and range in size form a few kilobases (kb) to hundreds of kb. Preferred cloning vectors have been modified from naturally occurring plasmids to facilitate the cloning and recombinant manipulation of polynucleotide sequences. Many such vectors are well known in the art and commercially available; see for example, by Sambrook (In. "Molecular Cloning: A Laboratory Manual," second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)), Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608(1980).

General and specific techniques for producing proteins from plant cells may be obtained from the following patents and applications, each of which is incorporated herein in its entirety by reference: U.S. Pat Pub. No. 2003/0172403 Al ("Plant Transcription Factors and Enhanced Gene Expression"); U.S. Pat. No. 6,991,824 ("Expression of Human Milk Proteins in Transgenic Plants"); U.S. Pat. Pub. No. 2003/0221223 ("Human Blood Proteins Expressed in Monocot Seeds"); U.S. Pat. Pub. No. 2004-0078851 ("Production of Human Growth Factors in Monocot Seeds"); U.S. Pat. Pub. No. 2004/0063617 ("Method of Making an Anti-infective Composition for Treating Oral Infections"); and international application no. PCT/US2004/041083 ("High- level Expression of Fusion Polypeptides in Plant Seeds Utilizing Seed- Storage Proteins as Fusion Carriers"). Other general and specific techniques for producing proteins from plant cells may be obtained, for example, from the following references, each of which is incorporated herein in its entirety by reference: U.S. Patent No. 5,693,507, U.S. Patent No. 5,932,479, U.S. Patent No. 6,642,053, and 6,680,426 (each titled "Genetic Engineering of Plant Chloroplasts"); U.S. Pat. Pub. No. 2005/0066384 ("Site-Targeted Transformation Using Amplification Vectors"): U.S. Pat. Pub. No. 2005/0221323 ("Amplification Vectors Based on Trans-Splicing"); U.S. Pat. Pub. No. 2006/0026718 ("Method of Controlling Cellular Processes in Plants"); and U.S. Pat. Pub. No. 2006/0075524 (Method of Controlling A Cellular Process in a Multi- Cellular Organism"); Marillonnet et at., Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants, Nature Biotech. (2005) 23(6): 718-723.

Expression vectors include plasmids, episomes, cosmids retroviruses or phages; the expression vector can be used to express a DNA sequence encoding the cell culture media component, and in one aspect comprises an assembly of expression control sequences. The choice of promoter and other regulatory elements can vary according to the intended host cell, and many such elements are available commercially, and can be readily assembled from isolated components such as the Gateway system from Invitrogen, (CA, USA). Expression systems for cell culture media components can be stable or transient expression systems.

Representative commercially available viral expression vectors include, but are not limited to, the adenovirus-based systems, such as the Per.C6 system available from Crucell, Inc., lentiviral-based systems such as pLP1 from Invitrogen, and retroviral vectors such as tobacco mosaic virus based vectors (Lindbo et al., BMC Biotechnol. (2007) 7 52-58).

An episomal expression vector is able to replicate in the host cell, and persists as an extrachromosomal episome within the host cell in the presence of appropriate selective

pressure. (See for example, Conese et al., Gene Therapy 11: 1735-1742 (2004)). Representative commercially available episomal expression vectors include, but are not limited to, episomal plasmids that utilize Epstein Barr Nuclear Antigen 1 (EBNA1) and the Epstein Barr Virus (EBV) origin of replication (oriP), specific examples include the vectors pREP4, pCEP4, pREP7 from Invitrogen. The host range of EBV based vectors can be increased to virtually any eukaryotic cell type through the co-expression of EBNA1 binding protein 2 (EPB2) (Kapoor et al., EMBO. J. 20: 222-230 (2001)), vectors pcDNA3.1 from Invitrogen, and pBK-CMV from Stratagene represent non-limiting examples of an episomal vector that uses T-antigen and the SV40 origin of replication in lieu of EBNA1 and oriP.

An integrating expression vector can randomly integrate into the host cell's DNA, or can include a recombination site to enable the specific recombination between the expression vector and the host cells chromosome. Such integrating expression vectors can utilize the endogenous expression control sequences of the host cell's chromosomes to effect expression of the desired protein. Examples of vectors that integrate in a site specific manner include, for example, components of the flp-in system from Invitrogen (e.g., pcDNA™5/FRT), or the cre-lox system, such as can be found in the pExchange-6 Core Vectors from Stratagene. Examples of vectors that integrate into host cell chromosomes in a random fashion include, for example, pcDNA3.1 (when introduced in the absence of T-antigen) from Invitrogen, pCI or pFN10A (ACT) Flexi® from Promega.

Alternatively, the expression vector can be used to introduce and integrate a strong promoter or enhancer sequences into a locus in the cell so as to modulate the expression of an endogenous gene of interest (Capecchi MR, Nat Rev Genet. (2005); 6 (6):507-12; Schindehutte et al., Stem Cells (2005); 23 (1):10-5). This approach can also be used to insert an inducible promoter, such as the Tet-On promoter (US Patents 5,464,758 and 5,814,618), in to the genomic DNA of the cell so as to provide inducible expression of an endogenous gene of interest. The activating construct can also include targeting sequence(s) to enable homologous or non-homologous recombination of the activating sequence into a desired locus specific for the gene of interest (see for example, Garcia-Otin & Guillou, Front Biosci. (2006) 11:1108-36). Alternatively, an inducible recombinase system, such as the Cre-ER system, can be used to activate a transgene in the presence of 4-hydroxytamoxifen (Indra et al. Nuc. Acid. Res. (1999) 27 (22): 4324-4327; Nuc. Acid. Res. (2000) 28(23): e99; and U.S. Patent No. 7,112,715).

Suitable cells for producing the cell culture media components include prokaryotic cells, yeasts, insect cells, plant expression systems and mammalian expression systems. Within these general guidelines, useful microbial hosts include, but are not limited to, bacteria from the genera Bacillus, Escherichia (such as *E. coli*), Pseudomonas, Streptomyces, Salmonella, Erwinia, *Bacillus subtilis*, *Bacillus brevis*, the various strains of *Escherichia coli* (e.g., HB101, (ATCC NO. 33694) DH5α, DH10 and MC1061 (ATCC NO. 53338)).

Many strains of yeast are known to those skilled in the art and are also available as host cells for the expression of cell culture media components including those from the genera Hansenula, Kluyveromyces, Pichia, Rhino-sporidium, Saccharomyces, and Schizosaccharomyces, and other fungi. Preferred yeast cells include, for example, *Saccharomyces cerivisae* and *Pichia pastoris*.

Additionally, where desired, insect cell systems can be utilized to produce the cell culture media components for use in the supplements and methods of the present invention. Such systems are described, for example, by Kitts et al., Biotechniques, 14:810-817 (1993); Lucklow, Curr. Opin. Biotechnol., 4:564-572 (1993); and Lucklow et al. (J. Virol., 67:4566-4579 (1993). Preferred insect cells include Sf-9 and HI5 (Invitrogen, Carlsbad, Calif.).

Many suitable plant expression systems can be used for the expression of cell culture media components examples includes for example, any monocot or dicot plant. Suitable monocot plants include without limitation, rice, barley, wheat, rye, corn, millet, triticale, or sorghum, preferably rice. Other suitable plants include Arabidopsis, Alfalfa, tobacco, peanut and soybean.

In general, expression vectors for use in plants include the following operably linked elements that constitute a chimeric gene: a promoter derived from a gene encoding a plant protein, operatively linked to a gene encoding a cell culture media component. The promoter region is chosen to be regulated in a manner allowing for induction under seed-maturation conditions. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, corn, millet, triticale or sorghum.

In one aspect, the chimeric gene includes a promoter which exhibits increased expression during seed maturation. Examples of such promoters include the maturation-specific promoter

region associated with one of the following maturation-specific monocot plant storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

In one aspect, the chimeric gene includes i) a promoter from the gene of a maturation-specific monocot plant storage protein, ii) a first DNA sequence, operably linked to the promoter, encoding a monocot plant seed-specific signal sequence (such as an N-terminal leader sequence or a C-terminal trailer sequence) capable of targeting a polypeptide linked thereto to an endosperm cell, preferably an endosperm-cell organelle, such as a protein storage body, and iii) a second DNA sequence, linked in translation frame with the first DNA sequence, encoding a cell culture media component. The signal sequence is preferably cleaved from the cell culture media component in the plant cell.

The chimeric gene, in turn, is typically placed in a suitable plant-transformation vector having (i) companion sequences upstream and/or downstream of the chimeric gene which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the desired plant host; (ii) a selectable marker sequence; and (iii) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells.

At least one selective purification tag and/or at least one specific protease cleavage site may be provided for eventual release of the cell culture media component from the monocot seed storage protein carrier. For example, a strategic methionine or tryptophan residue providing a chemical cleavage site may be engineered in frame between the domains for release of the cell culture media component from the endogenous plant protein. Other selective protease cleavage sites include, but are not limited to enterokinase (ek), Factor Xa, thrombin, V8 protease, GENENASE™(a variant of subtilisin BPN'), α-lytic protease or tobacco etch virus protease. Alternatively, cleavage of the fusion protein could be performed via chemical cleaving agents such as cyanogen bromide or N-chlorosuccinimide.

A number of suitable mammalian host cells are also known in the art and many are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209. Examples include, but are not limited to, mammalian cells, such as Chinese

hamster ovary cells (CHO) (ATCC No. CCL61) CHO DHFR-cells (Urlaub et al., Proc. Natl. Acad. Sci. USA, 97:4216-4220 (1980)), human embryonic kidney (HEK) 293 or 293T cells (ATCC No. CRL1573), or 3T3 cells (ATCC No. CCL92). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 (ATCC No. CRL1650) and COS-7 cell lines (ATCC No. CRL1651), and the CV-1 cell line (ATCC No. CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines.

Cell-free transcription and translation systems can also be employed to produce such proteins using the DNA constructs (or RNAs derived from the DNA constructs) of the present invention.

Production of recombinant proteins of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides encoding the cell culture media component and to host cells which are genetically engineered with such expression systems and to the production of such proteins by recombinant techniques. In one embodiment the host cell endogenously expresses a heat shock protein of interest.

In cases where purification of the expressed proteins of the supplement of the invention are necessary, proteins of the present invention can be recovered from either the cellular environment, before lysing the cells, or after cell lysis. The proteins can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. High performance liquid chromatography is also employed for purification.

A search of patents, published patent applications, and related publications will provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying albumin. For example, US Patent Nos. 4,075,197; 4,086,222; 4,093,612; 4,097,473; 4,136,094; 4,228,154; 5,250,662; 5,656,729; 5,677,424; 5,710,253; 5,728,553; 5,994,507; 6,001,974; 6,638,740; 6,617,133 and 7,423,124 disclose various processes for purifying

albumin. In one aspect, the albumin for use in the present invention is purified using any of these art recognized processes listed above, and then mixed in aqueous solution with a heat shock protein. Methods for the purification of heat shock proteins, including anion exchange chromatography and ATP agarose affinity chromatography are well known in the art. (Welch & Feramisco, J. Biol. Chem. 257 (24)14949-14959; (1982); Welch & Feramisco, Mol. Cell. Biol. 5 (6) 1229-1237 (1985). Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification.

In one preferred aspect recombinant albumin is purified using procedures that enable the direct co-purification of both recombinant albumin and a heat shock protein, or hsp protein complex, for example as described below and in the Examples section. In one aspect the recombinant albumin is produced in rice, and the heat shock protein is an endogenous rice heat shock protein.

In one aspect, of this method, expression vectors are used to increase the expression of the recombinant albumin in the host cell, while the expression of the host cells endogenous heat shock proteins is accomplished by activating the expression of the host cells hsp genes. In another aspect expression vectors are used to increase the expression of the heat shock protein. In another aspect expression vectors are used to increase the expression of the heat shock protein and albumin. In another aspect the nucleic acid sequence encoding a heat shock protein and albumin are located in the same expression vector.

Due to the similar electronegativity of albumin and hsp70, anion exchange chromatography is the preferred method to prepare albumin enriched in Hsps. For example, both albumin and Hsp70 bind to anion exchange columns with resins consisting of either quaternary amine or diethylaminoethyl mounted on a bead that is suitable for the ion exchange of polypeptides (large molecular exclusion limit and of suitable size) at high pH (7.5 and above). Examples of such resins are General Electric (GE) Q Sepharose and GE DEAE Sepharose. Due to their similar electronegativity, utilizing low pH conditions (below pH 6.5) allows for the co-purification of the two molecules on cation exchangers as well. Examples of such cation exchangers are GE Carboxymethyl Sepharose and Sulfonic acid Sepharose based resins. Because the albumin and Hsp70 have similar isoelectric points, mixed mode resins may also be employed for the co-purification of albumin and Hsp70. Since both Hsp70 and Albumin are well known to bind to

fatty acids and other hydrophobic molecules, it is also possible to co-purify albumin and Hsp70 on a hydrophobic based resin such as octyl sepharose (GE). Due the similar size of Hsp70 proteins and Albumin (65-75 kDa), co-purification of the two proteins and enrichment of Hsp70 by tangential flow ultrafiltration utilizing both higher and lower molecular exclusions than 65-75 kDa may also be employed to co-purify and thus enrich Albumin with hsps.

Also due to their similar molecular weights, any method that separates polypeptides based on size should effectively co-purify albumin and hsp70 such as molecular sieves and gel filtration or size exclusion chromatography. In addition, due to the similar nature of Hsp70 and Albumin in terms of hydrophobicity and electronegativity or surface charge may be co-purified by precipitation under a number of conditions. Some of those conditions are precipitation by ammonium sulfate, precipitation by denaturants such as urea, or precipitation based on isoelectric point and solubility.

The methods are also applicable to enrich albumin with hsps from other sources. For example albumin derived from native and transgenic animal feedstock serum, as well as albumin produced from recombinant organisms and tissue culture systems based on prokaryotic and eukaryotic cells, including, vertebrate cells such as mammalian cells, and non vertebrate cells, such as insects, as well as plant, and fungi such as yeast, and the like.

A search of patents, published patent applications, and related publications will also provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying transferrin related proteins. For example, US Patent Nos 7,368,141; 7,354,902; 5,708,149; 5,596,082; 5,169,936; 4,791,193; and 4,667,018 disclose various processes for purifying transferrin related proteins.

IV Exemplary Cells

Without wishing to be bound by theory, it is contemplated that any cell which is susceptible to apoptosis may be used in the methods of the invention, including primary cells, immortalized cells, differentiated cells, undifferentiated cells or cells, such as stem cells, with varying degrees of specialization. In a particular embodiment, cells used in the methods of the invention are transfected with a nucleic acid molecule comprising a nucleotide sequence encoding a protein of interest, e.g., a therapeutic protein or an antibody.

In a particular embodiment, the cells used in the methods of the invention are eukaryotic cells, e.g., mammalian cells. Examples of mammalian cells include, but are not limited to, for example, human B-cells, and T cells, and derivatives thereof, such as hybridomas, and cell expressing markers of B or T cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); CHO-K1 cell (ATCC CCL-61), human PER.C6 cells (Crucell, NV), mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; FS4 cells; NSO mouse myeloma cells (ECACC; SIGMA), and a human hepatoma line (Hep G2). Additional examples of useful cell lines include, but are not limited to, HT1080 cells (ATCC CCL 121), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB carcinoma cells (ATCC CCL 17), 2780AD ovarian carcinoma cells (see Van der Blick, A. M. et al., Cancer Res. 48:5927-5932 (1988), Raji cells (ATCC CCL 86), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells (ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607), WI-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. These and other cells and cell lines are available commercially, for example from the American Type Culture Collection (Virginia, USA). Many other cell lines are known in the art and will be familiar to the ordinarily skilled artisan; such cell lines therefore can be used equally well in the methods of the present invention. In a particular embodiment, cells used in the methods of the invention are CHO cells or NSO cells. Hybridomas and antibody-producing cells may also be used in the methods of the invention.

In another embodiment, cells used in any of the methods of the invention are stem cells. Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self renewing progenitors, non-

renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate in vitro into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts.

Types of human embryonic stem cells that may be used in any of the methods of the invention include established lines of human embryonic cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.). In one embodiment, Human embryonic stem cells are prepared as described by Thomson et al. (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995).

Additionally, hybridoma cells can also be used in the methods of the invention. The term "hybridoma" refers to a hybrid cell line produced by the fusion of an immortal cell line of immunologic origin and an antibody producing cell. The term encompasses progeny of heterohybrid myeloma fusions, which are the result of a fusion with human cells and a murine myeloma cell line subsequently fused with a plasma cell, commonly known as a trioma cell line. Furthermore, the term is meant to include any immortalized hybrid cell line which produces antibodies such as, for example, quadromas. See, e.g., Milstein et al., Nature, 537:3053 (1983). The hybrid cell lines can be of any species, including human, rabbit and mouse.

In some embodiments, a cell line used in the methods of the invention is an antibody-producing cell line. Antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan. See, e.g., Current Protocols in Immunology, Coligan et al., Eds., Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991)

which is herein incorporated by reference in its entirety, including supplements. In general, any cell suitable for recombinant protein expression in cell culture can be used in the methods of the invention.

In some embodiments, the cells used in the methods of the present invention may include a heterologous nucleic acid molecule which encodes a desired recombinant protein, e.g., a therapeutic protein or antibody which is desired to be produced using the methods of the invention. In a particular embodiment, the methods of the present invention are useful for producing high titers of a desired recombinant protein, e.g., a therapeutic protein or antibody, in the presence of reduced levels of one or more contaminants.

V. Cell Culture Media

Any suitable culture medium or feed medium suitable for cell growth and protein production may be used in the methods of the invention. Suitable culture or feed mediums are chosen for their compatibility with the host cells and process of interest. Suitable culture or feed mediums are well known in the art and include, but are not limited to, commercial media such as Ham's F10 (SIGMA), Minimal Essential Medium (SIGMA), RPMI-1640 (SIGMA), and Dulbecco's Modified Eagle's Medium SIGMA) are suitable for culturing the animal cells. In addition, any of the media described in Ham and Wallace, (1979) Meth. Enz., 58:44; Barnes and Sato,(1980) Anal. Biochem., 102:255; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469 or 4,560,655; International Publication Nos. WO 90/03430; and WO 87/00195 may be used.

Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The necessary growth factors for a particular cell are readily determined empirically without undue

experimentation, as described for example in Mammalian Cell Culture (Mather, J. P. ed., Plenum Press, N.Y. (1984), and Barnes and Sato, Cell, 22:649 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the protein of interest in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

VI. Exemplary Cell Culture Expression Products

In one aspect of any of the claimed methods, the supplements of the invention are used to improve the viability and growth of a cell which is used to express and produce a protein of interest. The cell may express the protein of interest endogenously or may be an engineered cell line that has been modified genetically to express the protein of interest at levels above background for that cell.

Cells may be genetically modified to express a protein by transformation with a nucleic acid encoding the protein of interest, or by transformation of an activating sequence that promotes the expression of an endogenous gene. In one aspect the protein of interest may be expressed from an expression vector, in which a coding sequence for the protein of interest is operably linked to an expression control sequences, to enable either constitutive or inducible expression, as is known in the art.

The protein of interest may be any protein, or fragment thereof, which is of commercial, therapeutic or diagnostic value including without limitation cytokines, chemokines, hormones, antibodies, anti-oxidant molecules, engineered immunoglobulin-like molecules, a single chain antibodies, a humanized antibodies, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, transdominant negative mutants of a target protein, toxins, conditional toxins, antigens, a tumor suppresser proteins, growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). The protein of interest may also comprise pro-drug activating enzymes.

In some embodiments, the protein of interest comprises a glycoprotein, or any other protein which has one or more post-translational modifications. For example, any protein which is suitable for production in a eukaryotic host may be expressed using the methods and compositions described here.

The methods of the invention can be used to produce any desired recombinant protein or fragment thereof. In some embodiments, a recombinant protein produced using the methods described herein is a therapeutic protein. In other embodiments, the recombinant protein is an antibody or functional fragment thereof. Antibodies which may be produced using the methods of the invention include, for example, polyclonal, monoclonal, monospecific, polyspecific, fully human, humanized, single-chain, chimeric, hybrid, CDR grafted,. It may comprise a full length IgG1 antibody or an antigen-binding fragments thereof, such as, for example, Fab, $F(ab')_2$, Fv, and scfv.

Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN™) (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN™), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108. B1, or Tositumomab (BEXXAR™.); anti-IL-8 (St John et al., Chest, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN™ (Kim et al., Growth Factors, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., Transplant Intl. 4:3-7 (1991), and Hourmant et al., Transplantation 58:377-380 (1994)); anti-IgE (Presta et al., J. Immunol. 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-alpha, antibodies including cA2 (REMICADE™), CDP571 and MAK-195 (See, U.S.

Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. J. Immunol. 156(4):1646-1653 (1996), and Dhainaut et al. Crit. Care Med. 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human alpha 4 beta 7 integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT™) and (ZENAPAX™) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al. Arthritis Rheum 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. Nature 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fc gamma RI as in Graziano et al. J. Immunol. 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. Cancer Res. 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. Cancer Res. 55(23): 5852s-5856s (1995); and Richman et al. Cancer Res. 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. Eur J. Immunol. 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. J. Immunol. 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. Cancer Res 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. Cancer Res 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX™.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO™ anti-RSV antibodies such as MEDI-493 (SYNAGIS™.); anti-CMV antibodies such as PROTOVIR™.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR™; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-.alpha.v.beta.3 antibody VITAXIN™; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

The recombinant protein may be a cellular protein such as a receptor (e.g., membrane bound or cytosolic) or a structural protein (e.g. a cytoskeleton protein). The recombinant protein may be cellular factor secreted by the cell or used internally in one or more signal transduction

pathways. Non limiting examples include, but are not limited to, CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1, IL-2, IL-3, IL-7, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF, EGF receptor, VEGF receptor, hepatocyte growth factor, osteoprotegerin ligand, interferon gamma, B lymphocyte stimulator C5 complement TAG-72, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2, and CTLA4 (which is a cytotoxic T lymphocyte-associated antigen).

The recombinant protein may also be derived from an infectious agent such as a virus, a bacteria, or fungus. The protein may be derived from a bacterial membrane or cell wall, or may be derived from the bacterial cytosol. The protein may be a bacterial or yeast enzyme, transcription factor, or structural protein. The bacterial or yeast protein may be membrane bound, cytsolic, or secreted. Examples of infectious agents include, but are not limited to, Streptococcus mutans, and Staphlycoccus aureus, and Candida albicans.

The methods of the invention can also be used to produce recombinant fusion proteins comprising all or part of any of the above-mentioned proteins. For example, recombinant fusion proteins comprising one of the above-mentioned proteins plus a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an antibody, or a substantially similar protein, can be produced using the methods of the invention. See e.g. International Application No. WO 94/10308; Lovejoy et al. (1993), Science 259:1288-1293; Harbury et al. (1993), Science 262: 1401-05; Harbury et al. (1994), Nature 371:80-83; Hang.kansson et al. (1999), Structure 7:255-64.

Also encompassed by this invention are pharmaceutical compositions including one or more recombinant proteins produced by the methods described herein. In some embodiments, pharmaceutical compositions further include a pharmaceutically acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a subject.

VII Stem Cells

In one embodiment, human embryonic stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation, comprising a supplement of the invention. The growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type and further comprising a supplement of the present invention. Alternatively, the growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a chemically defined medium comprising a supplement of the present invention. Examples of feeder-free, serum free culture systems in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal include those disclosed in US patent applications. US20050148070, US20050244962, US20050233446, U.S. Pat. No.6,800,480, and PCT publications WO2005065354 and WO2005086845.

In an alternate embodiment, human embryonic stem cells are initially cultured with a layer of feeder cells that support the human embryonic stem cells and further comprising a supplement of the present invention. The human embryonic are then transferred to a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation and which further comprises a supplement of the present invention. In any of these approaches, the use of the supplements of the invention results in significantly enhanced rates of cell growth and improved cell viability.

Examples of conditioned media suitable for use with the supplements of the present invention are disclosed in US20020072117, U.S. Pat. No. 6,642,048, WO2005014799, and Xu et al (Stem Cells 22: 972-980, 2004). An example of a chemically defined medium suitable for use with the supplements of the present invention may be found in US20070010011.

Examples of feeder cells include feeder cells selected from the group consisting of a fibroblast cell, a MRC-5 cell, an embryonic kidney cell, a mesenchymal cell, an osteosarcoma cell, a keratinocyte, a chondrocyte, a Fallopian ductal epithelial cell, a liver cell, a cardiac cell, a bone marrow stromal cell, a granulosa cell, a skeletal muscle cell, a muscle cell and an aortic endothelial cell. In a preferred embodiment, the MRC-5 cell, has ATCC Catalog Number 55-X;

the transformed and has ATCC Accession Number CRL-2309; the human osteosarcoma cell has ATCC Accession Number HTB-96; and the mesenchymal cell is a human fetal palatal mesenchymal cell with ATCC Accession Number CRL-1486. In other preferred embodiments the human fibroblast cell is a skin keloid fibroblast, KEL FIB and has ATCC Accession Number CRL-1762, or is a fetal skin fibroblast cell; and the bone marrow stromal cell, HS-5, has ATCC Accession Number CRL-11882.

Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco #15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

In one embodiment, the human embryonic stem cells are plated onto a suitable culture substrate that is treated prior to treatment according to the methods of the present invention, with a composition comprising a supplement of the present invention. In one embodiment, the treatment is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL (Becton Dickenson). MATRIGEL is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

Other extracellular matrix components and component mixtures are suitable as an alternative and can be used with the supplements of the present invention. This may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations with a supplement of the present invention.

In another embodiment, the invention encompasses an embryonic stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention. In one embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention.

In another embodiment the invention encompasses an embryonic stem cell culture, comprising a human embryonic stem cell and a human feeder cell culture comprising a supplement of the invention. In another embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a human feeder cell culture comprising a supplement of the invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing pluripotency markers, comprising the steps of:

Culturing human embryonic stem cells,

Differentiating the human embryonic stem cells into cells expressing pluripotency markers, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing markers, characteristic of ectodermal, endodermal or mesodermal cells, comprising the steps of:

Culturing pluripotency stem cells:

a. Differentiating the pluripotency stem cells into cells expressing markers characteristic of ectodermal, endodermal or mesodermal cells, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In any of these methods, the stem cells can be differentiated into cells expressing markers characteristic of an endodermal, ectodermal or mesodermal lineage by any method in the art. For example, cells expressing pluripotency markers may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 23, 1534-1541 (2005), by Shinozaki et al, Development 131, 1651-1662 (2004), McLean et al., Stem Cells 25, 29-38 (2007), D'Amour et al., Nature Biotechnology 24, 1392-1401 (2006).

Cells expressing markers characteristic of the endoderm lineage may be further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art. For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 24, 1392-1401 (2006), wherein the differentiation is conducted in the presence of a supplement of the present invention.

In one aspect of any of these methods of differentiation, the human embryonic stem cells are cultured and differentiated on a tissue culture substrate coated with an extracellular matrix. The extracellular matrix may be a solubilized basement membrane preparation extracted from mouse sarcoma cells (which is sold by BD Biosciences under the trade name MATRIGEL). Alternatively, the extracellular matrix may be growth factor-reduced MATRIGEL. Alternatively, the extracellular matrix may be fibronectin. In an alternate embodiment, the human embryonic stem cells are cultured and differentiated on tissue culture substrate coated with human serum. In one aspect, the tissue culture substrate is coated with extracellular matrix and a supplement of the present invention.

The extracellular matrix may be diluted prior to coating the tissue culture substrate. Examples of suitable methods for diluting the extracellular matrix and for coating the tissue culture substrate may be found in Kleinman, H. K., et al., Biochemistry 25:312 (1986), and Hadley, M. A., et al., J. Cell. Biol. 101:1511 (1985).

In one aspect of the methods of stem cell differentiation, the culture medium should contain sufficiently low concentrations of certain factors to allow the differentiation of human embryonic stem cells to cells of endoderm, ectoderm or mesoderm lineage, such as, for example insulin and IGF (as disclosed in WO2006020919). This may be achieved by lowering the serum concentration, or alternatively, by using chemically defined media that lacks insulin and IGF. Examples of chemically defined media are disclosed in Wiles et al (Exp Cell Res. 1999 Feb. 25; 247(1): 241-8.). In a preferred embodiment, of any of these methods, the culture media comprises a supplement of the present invention.

The culture medium may also contain at least one other additional factor that may enhance the formation of cells expressing markers characteristic of endoderm, mesoderm or ectoderm

lineage from human embryonic stem cells. The at least one additional factor may be, for example, nicotinamide, members of TGF-β family, including TGF-β1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (GDF-5, -6, -8, -10, 11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS, noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine 1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof. In a preferred embodiment, of any of these methods, the culture media containing at least one additional factor listed above, further comprises a supplement of the present invention.

The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No: HTB-79), BxPC-3 (ATCC No: CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241). In a preferred embodiment, of any of these methods, the conditioned media further comprises a supplement of the present invention.

In another embodiment, the invention encompasses a method of using the cell or tissue of any of the aforementioned stem cells for the experimental, therapeutic and prophylactic treatment of a disease or condition in a human or animal. Preferably, the disease is selected from the group consisting of Parkinson's, Alzheimer's, Multiple Sclerosis, spinal cord injuries, stroke, macular degeneration, burns, liver failure, heart disease, diabetes, Duchenne's muscular dystrophy, osteogenesis imperfecta, osteoarthritis, rheumatoid arthritis, anemia, leukemia, breast cancer, solid tumors, and AIDS. In a preferred embodiment, the disease is Parkinson's or Alzheimer's. In a more preferred embodiment, the disease is Parkinson's.

VIII. Large Scale production of recombinant proteins

In one embodiment, the supplements of the present invention can be used to produce a protein of interest by growing host cells in the presence of the supplement. In one embodiment, the cell culture is performed in a stirred tank bioreactor system and a fed batch culture procedure is employed. In another embodiment a wave disposable bioreactor is employed. In the bioreactor system, the size of the bioreactors are sufficiently large to produce the desired amount of protein of interest, such as 1,000 Liter or 12,000 Liter sizes, but are not limited to such sizes as much smaller (i.e., 2 Liter, 400 Liter) or larger (i.e., 25,000 Liter, 50,000 Liter) bioreactor vessels may be appropriate. In the preferred fed batch culture, the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process but at the termination of the culture process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cultured cells may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single step or multiple step culture procedure. In a single step culture, the host cells are inoculated into a culture environment and the method steps of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture, cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium comprising a supplement of the present invention suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In

the growth phase, cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 38° C. and preferably about 37° C. and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above, the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell culture environment during the production phase of the cell culture is controlled. According to the steps of the presently disclosed methods, the addition of the supplements of the invention can be coordinated such that the desired content and quality of the protein of interest is achieved and maintained in the resulting cell culture fluid. In a preferred aspect, the production phase of the cell culture is preceded by a transition phase of the cell culture in which the addition of the supplements of the invention initiates the production phase of the cell culture.

In any of the above-described methods, it is contemplated that it may be desirable to include a desired amount of agent like butyrate or Trichostatin A in the cell culture medium in combination with a supplement of the invention. Various forms of butyrate and its salts are known in the art, such as butyric acid and sodium butyrate, and are publicly available from sources such as Sigma Chemical Co. Butyrate has been reported in the literature to enhance the productivity and protein expression of cell cultures [Arts et al., Biochem J., 310:171-176 (1995); Gorman et al., Nucleic Acids Res., 11:7631-7648 (1983); Krugh, Mol. Cell. Biochem. 42:65-82 (1982); Lamotte et al., Cytotechnology, 29:55-64 (1999); Chotigeat et al., Cytotechnology, 15:217-221 (1994)]. Trichostatin A (TSA) is an inhibitor of histone deacetylase and may act similarly to butyrate in enhancing the productivity and protein expression in cell cultures [Medina et al., Cancer Research, 57:3697-3707 (1997)]. Although butyrate has some positive effects on protein expression, it is also appreciated in the art that at certain concentrations, butyrate can induce apoptosis in the cultured cells and thereby decrease viability of the culture as well as viable cell density [Hague et al., Int. J. Cancer, 55:498-505 (1993); Calabresse et al., Biochim. Biophys. Res. Comm., 195:31-38 (1993); Fillipovich et al., Biochim. Biophys. Res. Comm., 198:257-265 (1994); Medina et al., Cancer Research, 57:3697-3707 (1997)]. In the methods of the present invention, a desired amount of butyrate or TSA may be added to the cell culture at

the onset of the production phase and more preferably, may be added to the cell culture after a temperature shift has been implemented. Butyrate or TSA can be added in a desired amount determined empirically by those skilled in the art, but preferably, butyrate is added to the cell culture at a concentration of about 1 to about 25 mM, and more preferably, at a concentration of about 1 to about 6 mM.

Expression of the protein of interest may be measured in a sample directly, for example, by ELISA, conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorophors or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Many are commercially available.

The supplements claimed herein can also be used to increase transfection efficiency and viability of cells during transfection. Conditions and reagents used in various transfection techniques, such as Lipofectamine are relatively toxic to the cells, while electroporation can severely stress a cell. The use of higher concentrations of transfection reagents, and more extensive electroporation conditions is preferred to achieve higher transfection efficiencies.

Thus the addition of the supplements of the invention prior, with, and after transfection can result in higher transfection efficiencies, and higher yields of recombinant proteins.

The supplements of the invention can be used to express proteins of interest which induce apoptosis, such as Apo-2 ligand / TRAIL or Fas ligand. The presence of the supplements of the invention may block such apoptotic activity and allow for improved expression of the protein of interest.

In addition, the methods can be used to increase the viability of cells undergoing freezing / storage/thawing procedures. During these procedures generally cells can lose viability. The presence of apoptosis inhibitors added to the cell culture media can provide for increased cell viability and aid in reducing or eliminating the variability in cell viabilities between aliquots or vials of cells.

IX. Kits

Also encompassed by the present invention are kits for promoting the viability of cells. In one embodiment, a kit according to the present invention comprises: (a) one or more reagents or devices for transfection and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding using the transfection device, transfection agent and supplement.

In another embodiment a kit according to the present invention comprises: (a) one or more reagents or devices for freezing or thawing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for freezing or thawing cell lines and the use of the reagents.

In another embodiment a kit according to the present invention comprises: (a) one or more tissue culture products for culturing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for dilution cloning techniques and the use of the reagents in such approaches.

All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and indicated individually to be incorporated by reference in its entirety. The following examples illustrate but are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Expression of recombinant human lactoferrin.

A. An Expression Vector For Human Lactoferrin Expression In Transgenic Rice

Complete nucleotide sequence of human mammary gland lactoferrin was codon optimized and synthesized by Operon Technologies (CA, USA). Human milk lactoferrin gene (Genbank accession number: HSU07642) was re-synthesized with codons most frequently used in translation of rice seed proteins in order to obtain optimal level of expression (**Figure 1**). Although numbers of codons changed accounted for 22.46% of the entire sequence, amino acid composition remains identical to native human lactoferrin. The plasmid containing the codon-optimized gene was called Lac-ger. Lac-ger was digested with SmaI/XhoI and the fragment containing the lactoferrin gene was cloned into pAPI141 that was partially digested with NaeI and completely digested with XhoI. For expression of hLF in rice seeds, the codon-optimized gene was operably linked to the rice endosperm-specific glutelin (Gt1) promoter and NOS terminator. The resulting plasmid was designated pAPI164 (**Figure 2**).

B. Production system

Rice variety Taipei 309 (Oryza sativa, Japonica) was selected as the production system for recombinant human lactoferrin and transgenic rice events were generated by the particle bombardment of embryogenic rice calli with the plasmid pAPI164 and a companion marker plasmid containing the hygromycin phosphotransferase gene as a selectable marker. Fully developed, fertile rice plants were obtained by this procedure.

C. High Level Protein Expression of recombinant human lactoferrin in rice grain

Expression of recombinant human lactoferrin was under the control of the seed maturation-specific promoter Gt1. The high level expression of recombinant human lactoferrin is evident in **Figure 3** as independent transgenic rice events were screened. Total soluble proteins from mature rice seed extracts were run on Laemli gels and stained with Coomassie blue to visualize the proteins. An ~80kD recombinant lactoferrin protein was obtained in all transgenic lines as indicated by the stained gel. Expression levels of recombinant human lactoferrin corresponded to 0.5% of seed weight. The stable expression of recombinant human lactoferrin was monitored

for 10 generations. The expression level is maintained at approximately 0.5% of seed weight of brown rice (**Figure 4**).

Example 2: Expression & purification of recombinant human transferrin.

Purification of recombinant human from rice grain.

The rice grain is dehusked and milled to an average of 100 mesh flour using standard food industry procedures. Hulls and the solid material remaining after the extraction described below are destroyed. Recombinant human transferrin is extracted from the flour with extraction buffer (20 mM Tris-Base, pH 7.5), with stirring, for 40 minutes at room temperature. Filter aid (Cellpure 300) is added at 20g/L to the extraction mixture before filtration through a filter press with 10 kDa regenerated cellulose.

The filter press filtrate was diafiltered and concentrated with a 30 KDa molecular weight cutoff cellulose membrane into10 mM Tris-Base pH. 8.5 to prepare for DEAE chromatography. The DEAE column was washed with (20mM Tris-Base, 40mM Sodium Chloride, pH 7.5) prior to loading the concentrate onto the column. After loading, the column was washed with 3 volumes of 10 mM Tris-Base, 0.5% (v/v) Triton X-114, pH 8.5 buffer followed by 7 volumes of 10 mM Tris-Base, pH 8.5 buffer. Transferrin was then eluded with 20mM Tris-Base, 40mM Sodium Chloride, pH 7.5.

Transferrin that was recovered from the DEAE column was diafiltered and concentrated with a 10 KDa molecular weight cut off cellulose membrane into 10 mM sodium phosphate, 25mM sodium chloride, pH 7.0 buffer. After diafiltration, recombinant transferrin was filtered though a 0.2 u filter prior to drying.

Example 3: Expression of recombinant human albumin in rice grain.

Protein sequences of human albumin (HSA) from various data bases were compared. The consensus sequence represented by accession number P02768 (Swiss-Prot) was used as a base for gene codon-optimization for suitable expression of human albumin in rice grain (**Figure 5**). Gene synthesis was carried out by Blue Heron (Seattle, WA) and the synthetic fragment was inserted into pUC based vector to create pUC-HSA. After confirmation of correct DNA and protein sequences, pUC-HSA was digested with MlyI and XhoI. The fragment containing

codon-optimized HSA gene was inserted into pAPI405, which had been precut with NaeI and XhoI. Plasmid API405 was a derivative of pAPI141 which included Gt1 promoter, Gt1 signal sequence and a nos terminator.

Insertion of MfyI/XhoI fragment into pAPI405 resulted in pAPI504 (**Figure 6**). Plasmid API504 was then cleaved with HindIII and EcoRI. The HindIII/EcoRI fragment containing the entire expression cassette, Gt1 promoter, Gt1 signal sequence, codon-optimized HSA gene and nos terminator, was cloned into pJH2600 predigested with the same enzyme resulting in pAPI508 (**Figure 7**). Plasmid JH2600 was a shuttle vector between E coli and Agrobacterium. After DNA sequence verification, pAPI508 was moved into Agrobacterium AGL-1 for rice transformation. Plasmid API504 was also used via bombardment-based transformation following the procedure described previously. Upon transformation, transgenic plants were generated and were sent to greenhouse where transgenic R0 plants grew to maturity and set R1 seeds.

To monitor the expression of HSA in rice seeds, 10 R1 seeds from each R0 plant were extracted using 10 mL of extraction butter (50 mM Tris-HCl pH 8.0, 150 mM NaCl). The supernatant was collected and the expression level from rice extracts was monitored by an ELISA (Bethyl Laboratories, Montgomery, TX). The results showed that the HSA expression level in rice transgenic seeds ranges from 0.01 to 0.85% of brown flour weight (0.1 to 8.5 grams/kg flour). The results of the eight events with highest expression levels are shown in the following table (**Table E1**).

Table E1. Recombinant HSA expression level in top eight events

Line Number	Express Level (g/kg flour)
508-30	5.5
508-17	8.0
508-71	8.7
508-73	4.0
508-77	8.5
508-83	8.5
508-101	3.0
508-113	4.0

Example 4: Purification of recombinant human lactoferrin as cell culture media component.

To prepare cell culture media supplemented with recombinant human lactoferrin, recombinant human lactoferrin was purified from rice flour. Transgenic rice line (164-12) expressing high levels of recombinant human LF was selected. This line, now named as LF164, was planted two generations per year, alternating field planting in summer and greenhouse planting in winter. For protein purification, paddy rice expressing rhLF was de-hulled (Rice Mill, PS-160, Rimac, FL), and then ground to flour (average particle size of 100 mesh) using a hammer mill (8WA, Schutte-Buffalo, NY).

Protein extraction from transgenic flour was performed by mixing two kg of rice flour and 20 L of extraction buffer (0.02 M sodium phosphate pH 6.5 and 0.3 M sodium chloride) in a 50 L tank for 1 h. At the end of the mixing period, the suspension was allowed to settle overnight or centrifuged at 3750 rpm. In both cases, the supernatant was filtered through a plate and frame filter press (Ertel Alsop, 8S, NY) using M-05 and M-70 cellulose/perlite-based filters (Ertel Alsop, NY), respectively.

The filtrate containing rhLF and other rice flour soluble proteins was loaded onto an ion exchange column for further purification. An INDEX 200/500 process column (Amersham Pharmacia Biotech, NJ) packed with SP-Sepharose fast flow (Amersham Pharmacia Biotech, NJ) was used. The column was run at a linear flow rate of 150-200 cm/h. Packing, cleaning and testing of the resin bed was executed per manufacturer's instruction (HETP Test). The filtrate was loaded on to the resin at a linear velocity of 175 cm/h and washed with 0.02 M sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl until the A_{280} returned to baseline. Recombinant hLF was eluted using 20 mM sodium phosphate buffer (pH 6.5) containing 0.8 M NaCl. The washing and elution were performed at 200 cm/h and 150 cm/h, respectively.

A Centramate module (Pall Biopharmaceutical, MA) with 1 ft^2 50 kDa polyethersulfone (Pall Biopharmaceutical, MA) membrane was used for concentration and desalting (ultrafiltration) of eluted hLF. The filtration was performed at a cross flow rate of approximately 1.5 L/min and an average transmembrane pressure of 10 psig. The eluted rhLF was concentrated and desalted to a final volume of 0.25 L and then lyophilized dry. Usually, about 3 grams of purified recombinant human lactoferrin was recovered from one kilogram of transgenic rice flour.

The recombinant human lactoferrin purified from rice flour is approximately 50% saturated with iron (partial-lactoferrin). The 50% saturated recombinant human lactoferrin was then made >90% iron saturated by iron up taking treatment, resulting in holo-lactoferrin and was made <10% iron saturated by acid treatment to remove bound iron resulting in apo-lactoferrin. The purified lactoferrins (holo-, partial- and apo-) were used as cell culture media components.

Example 5: Purification and characterization of recombinant serum human albumin (rHSA).

Overview of B000 production process.

To purify rHSA from rice grain, rice grain expressing recombinant human serum, as described above, was dehusked, ground to flour and mixed for 30 min. with rHSA extraction buffer (Sodium Acetate, pH 4.9). After clarification by filtration, the filtrate was loaded onto an anion-exchange column (Q-Sepharose; GE Healthcare) at a rate of approximately 150 cm/hr. Once loaded, the resin was then washed with equilibration buffer to remove any unbound protein. The eluted rHSA is approximately 90% pure as analyzed by SDS-PAGE. Blue-Sepharose (GE Healthcare) can then be used to increase the purity from 90% to approximately 98% based on scanning analysis of SDS-PAGE gels. The rHSA is then concentrated and desalted via ultrafiltration prior to freeze drying.

Purified recombinant HSA was subjected to a range of biochemical and biophysical characterizations for comparison to pHSA (Plasma-derived HSA) (**Table E2**). These tests indicate that rHSA expressed in rice is equivalent to pHSA.

Table E2

Properties of recombinant albumin (rHSA) as compared to plasma derived albumin (pHSA)

Property	pHSA	rHSA
N-terminal sequence	DAHKSE	DAHKSE
Glycosylation	None	None
SDS-PAGE/Western	~66 kDa	~66 kDa
Molecular mass (MALDI)	66.9-68.1 kDa	66.8-67.9 kDa
Isoelectric focusing point	pI 5.3	pI 5.3
Ligand binding	Yes	Yes

Thermal stability	Midpoint 65°C	Midpoint 65°C
Esterase activity	Yes	Yes
Protease sensitivity	Same as each other	Same as each other

B0000C Production Process: For Ventria grown rice, the rice was harvested by combine or by hand. During this process the mature seeds were separated from the vegetative plant matter by the combine separator or by manual labor. The harvested rice was dried to approximately 12% moisture at which point it is suitable for storage in a clean grain bin, storage tote, supersack, or other container that will protect the grain from birds, rodents, lizards, insects and other pests. When the rice grain is needed for flour, it is first dehusked or dehulled. This process is done under vacuum such that debris and the outer part of the seed are swept away from the endosperm and germ or bran layer. The dehusked grain is then either washed and dried, or washed and processed directly as in wet homogenization, or processed further in the dry, dehusked state. The dry, dehusked material may be debranned by a rice polishing or debranning machine which are common to white rice producers.

Debranned, dehusked rice may be washed at this point and wet-milled or dried for dry milling or processed directly by grinding into flour. Milling with the least amount of shear and heat is preferred as such with a roller mill or pin mill. A hammermill is also suitable. The flour should be ground such that the protein can be extracted to 90% in less than 5 minutes in water with hard agitation. Normally that requires a size of particle that is smaller than 400 micrometers or 4 mm. However, larger particles can be extracted if given longer time. Alternatively, the grain can be washed and wet milled with a liquid homogenizer set up such that 90% of the extractable protein is solubilized.

The flour slurry is typically mixed at a ratio of at least 3 parts water to 1 part flour and up to 20 parts water to 1 part flour. The water typically contains suitable buffers such as Tris/HCl, Citrate, Phosphate, HEPES, or the like, such that the pH is maintained around pH 7 and a small amount of salt such as 100 mM NaCl. After the slurry is homogenized in the case of wet milling, or mixed thoroughly for dry flour, the bulk solids are removed from the slurry by way of solid liquid separation. This is carried out by decanting, centrifugation, or filtration; for example using plate and frame with pads, pressure filter, belt filter, vacuum flask, hydroclone, or vacuum belt filter. After filtration, the compressed cake should be washed with extraction buffer to recover protein from the cake. The addition of diatomateous earth or other filter media is useful in promoting the clarity of the filtrate but is not necessary given the right equipment. Alternatively, a flocculating agent may be used to aid in clarification. The clarified filtrate should be checked

for its albumin content and verified that the recovery is consistent with the determined expression level in the rice seed.

In order to remove starches, precipitable proteins, viruses, and other contaminants, 5 M acetic acid is added to the clarified filtrate until the pH reaches 5.0 and the solution turns white. The white solution is agitated for at least 20 minutes to encourage precipitation of insoluble materials. The precipitated solution is then filtered through a depth filter, such as a canister filter, cartridge filter or other filtration device to reach an optical clarity that is suitable for ultrafiltration, or less that 10 NTU (nephelometry turbidity units). It can also be clarified with a filter press, pressure filter, or alternatively by using a ceramic filter or other material that utilizes cross-flow. In addition, this material is suitable for direct application to an expanded bed chromatography column.

In a preferred method, the clarified filtrate is clarified via filtration through a 0.2 micron filter, and neutralized to pH 7.0 with 1M NaOH. This material is then suitable for ultrafiltration by hollow fiber, flat sheet, or spiral wound cross flow filtration. The material can be passed through a membrane of 100 kilodalton (kDa) size or larger to remove viruses, unwanted larger contaminants, and aggregates. The material that passes through the membrane can be concentrated by a 10 or 30 kDa crossflow membrane and then the same membrane can be used to prepare the solution for chromatography. The concentrated material can then diafiltered with column equilibration buffer until the conductivity and the pH are equalized.

The preferred buffer for anion exchange chromatography on GE DEAE sepharose or GE Q sepharose is 10 or 20 mM Tris/HCl buffer pH balanced to pH 8.0. In contrast, the preferred buffer for cation exchange, for example via the use of for negatively charged resins or negatively charged resins mixed with a hydrophobic linker (mixed mode absorbents), or alternatively blue Cibicron such Blue Sepharose (GE) is acetate or citrate buffer pH balanced to 4.8 to 5.0

For either system the albumin and other similarly charged proteins will be retained by the matrix and washing is conducted to remove loosely bound material by washing with at least 5 column volumes of loading buffer, which may also include detergents as deemed necessary to help remove hydrophobic impurities. The material can be eluted by charging the column with the same or modified buffers with the pH increased 2-4 units for cation exchange or decreased 2-4 units for anion exchange. The resulting change in pH will allow for the exchange of ions and the protein will be eluted in a sharp band. To increase the purity of the elution fraction, the elution peak can be scrutinized such that the first portion (10%) or last (10%) or both portions can be excluded from the main elution peak. In the preferred method, a solution containing phosphate

at 100 mM and pH adjusted to pH 4.0 including 10 mM NaCl is used to elute the protein from GE Q sepharose (Fast Flow). In this instance, pH and conductivity are used to elute the material allowing the discrimination between non-binding contaminants (flow through and wash) and tighter binding contaminants (those that are retained on the column in 100 mM Phosphate, 10 mM NaCl, and pH adjusted to 4.0).

After elution, if the pH of the eluted material has a pH of less than 6.0, then it is neutralized with 1M NaOH. The resulting solution is then diafiltered against the same buffer for the next chromatography step, which in a preferred method involves flowing the elutent through a column of the same matrix (i.e. Q sepharose) except in the non-binding mode with 100 mM Phosphate, 10 mM NaCl, and pH 7.0.

The second column step uses the same principles as the first but in reverse mode such that the contaminants that were co-eluted on the binding column have an opportunity to be retained on the matrix at a neutral pH. The flow through material from the first capture column can also be treated with a variety of alternative types of chromatography approaches, for example, cation exchange, hydrophobic, mixed mode, or gel filtration chromatography.

In a preferred method, the flow through material from the Q sepharose non-binding column is concentrated on a 10 kDa or 30 kDa crossflow membrane until the concentration is between 15 and 25% albumin. The buffer is then changed by diafiltration into a suitable buffer for cell culture such as Dulbeccos PBS or alternatively 20 mM Phosphate, 50 mM NaCl, and pH 7.0. The material is then sterile grade filtered into a sterile container. The sterile filtered material may be treated with detergent to destroy enveloped viruses and to aid in the removal of hydrophobic toxins and contaminants. In a preferred method, 0.5% v/v Triton X-114 or X-100 is added to the 15 to 25% albumin solution at room temperature (less than 23 C and greater than 18 C) and the solution is agitated or stirred for at least hour. The material is then passed over a hydrophobic resin with a molecular weight exclusion limit that is much less than the molecular weight of albumin. Many commercially available resins are available including those from Biorad and Pall Corporation.

The material that is passed over the column may then be tested in cells that are sensitive to detergent to confirm biological activity. The residual detergent that remains should typically be less than 0.005% with respect to the albumin solution. The detergent free flow through can then be sterile filtered into containers for direct shipment, or can have stabilizers added, or can be subjected to pasteurization with stabilizers, or can have stabilizers added before drying or dried directly. The material may be dried by lyophilization or spray drying. Prior to drying, in some instances, it may be useful to subject the material to a virus filtration step using a disposable,

validated, virus removing capsule such as is available from GE, Pall, and Millipore. It is common in the art to understand that a prefiltration step may be necessary in order to effectively and economically pass the concentrated material through a 20 nm filter.

Results: Rice flour was extracted at 1:5 ratio in phosphate buffered saline and mixed for 20 minutes. The liquid was clarified using a Nalgene filter flask. The subsequent clarified extract was subjected to acid precipitation as is described in the methods. The solution was then filtered and neutralized to give a clarified filtrate. This material was diafiltered against 50 mM Tris/Cl pH 8.0 until the material and buffer were equilibrated. The material was then loaded (300-600 cmh) on a pre-equilibrated GE Q-sepharose column to allow for 50 g/L binding capacity. The loaded material was washed with the same buffer and the material was then eluted with 100 mM Phosphate, 10 mM NaCl, and pH 4.0 as described above. The material eluted in a sharp peak and the collected eluate had a stable pH of about 5.8. Albumin produced using this method was compared to other sources of Albumin as more fully disclosed below: The eluate was collected in a pool and 1M NaOH was added until the pH was greater than 6.0. The material was then concentrated on a 10 kDa regenerated cellulose membrane approximately 5 fold and approximately five equal volume diafiltrations were carried out with 100 mM phosphate, 10 mM NaCl, pH 7.0. The final diafiltered material was checked for albumin protein content (in relation to the expression level in the starting material should be greater than 80%) and endotoxin level (should be less than 100 EU/mg depending on the feed material). This material was passed (60-160 cmh) over a Q-sepharose column, equilibrated with 100 mM phosphate, 10 mM NaCl, pH 7.0, of sufficient size to allow for approximately 2-3 times loading volume. The material was washed through the resin with the same buffer and collected. The collected material was diafiltered on a 10 kDa regenerated cellulose membrane and concentrated approximately 10 fold or until the albumin concentration reaches at least 10% or not more than 20% and five equal volume diafiltrations were performed with 20 mM phosphate, 50 mM NaCl, pH 7.0. After sterile grade filtration (0.2 µm), the solution was agitated for 1 hour with 0.5% (v/v) Triton X -100 at 20 +/- 2°C. After the incubation, the material was passed through Pall SDR resin according to the manufacturer's directions. The flow through material was sterile grade filtered into sterile containers and refrigerated or freeze dried as is common for protein and salt solutions.

Example 6: <u>Comparison of recombinant albumin produced from rice using B0000C process compared to other sources of albumin and previous methods for the production of Albumin.</u>

Methods: Albumin prepared using process B000C described in Example 5, was compared to albumin prepared using an alternative process (B000) which was previously used to prepare recombinant albumin (Cellastim (Batches B202 to B217)).

Albumin production (old process, B000):

Rice flour and 25 mM Sodium phosphate, 50 mM Sodium Chloride, was pH balanced to 6.5 with NaOH and mixed for 20 minutes at room temperature with a S/L ratio of approximately 1:10. Filter aid (Cellpure 300) was added at 10 g/L and the slurry was filtered by filter press, vacuum filtration, or centrifugation. The clarified filtrate was acid precipitated to about pH 5.0 with 1 M acetic acid. The resulting solution was filtered as described above with the addition of 5 g/L filter aid (Cellpure 300). The material was neutralized immediately to pH 6.5 to 7.0 with 1M NaOH. The material was diafiltered (10 kDa regenerated cellulose for all UFDF steps) with 5 equal diavolumes of the same buffer used for extraction. The material was loaded on a pre equilibrated Q-sepharose column (GE Healthcare) to allow for 8 g albumin binding per liter of resin at 60 cmh.

After washing the column with 5 column volumes of the same buffer, the albumin was eluted by increasing the salt concentration to 250 mM NaCl in one step. The resulting material was diafiltered against 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0 with 5-7 equal diavolumes. The resulting material was passed over a Q-sepharose column equilibrated with the 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0, and collected as flow-through. The flow-through material was then concentrated and diafiltered against 20 mM sodium phosphate, 10 mM NaCl, pH 7.0 with 5 diavolumes. The final concentrated material was sterile filtered and incubated with 10 g/L of the detergent CHAPS ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid) and mixed at room temperature for 1 hour. After the one hour incubation, the material was passed over a Biorad SM-2 column. The material was sterile filtered and freeze dried.

Size Exclusion Chromatography analysis.

Purity analysis by HPLC was carried out in 100 mM phosphate, pH 7.0 on a GF-250 column (Agilent Technologies) at a flow rate of 1 ml/min with the detector set at 214 and 280 nm. A standard curve was developed by injecting 5 different dilutions made by dry powder with a correction factor of 0.92 for salt and moisture. The main peak from 214 nm was integrated either by retention time or alternatively baseline. The unknown sample was injected at a concentration that is within the range of the standard injections. The unknown concentration of

albumin per dry powder weight (purity) was calculated from the standard curve. In a typical experiment, the 0, 5, 8, 10, 15, and 20 µg of the standard was injected followed by approximately 10 µg of unknown sample in approximately 50 µL injection volume. The correlation coefficient for the standard curve after integrating the peaks was typically above 0.98.

SDS PAGE and Densitometry: (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Samples were prepared by diluting the protein solutions to 1-2 mg/ml to enable a defined amount of each protein to be loaded on to each well. The sample was mixed 1:1 with Tris-Glycine SDS sample buffer (LC2673 Novex) containing reducing agent (Invitrogen NP0004) and heated to 70 °C for 5 minutes. The sample was loaded (10, 20, or 30 µg) onto a Novex 4-20% precast gel and separated at constant voltage (130V) in standard Tris-Glycine-SDS running buffer. The electrophoresis was ended when the tracking dye reached the end of the gel. A molecular weight marker was included in the first lane as a reference.

The gel was stained with G Bioscience (786-35G) and destained with water. A digital image was obtained with a Hewlett Packard Scanner (G4010). The image file was then opened with UN-SCAN-IT (Silk Scientific Corp.). The densitometry was carried out with positive image analysis in 256 grayscale in which all visible bands were included as individual segments. The background noise was corrected by four corner interpolation as specified in the software for each segment. The signal for each segment or band was then calculated from the product of the # of pixels and the average pixel intensity (0-255). The sum of the signals for an entire lane (all visible segments or bands) was taken as 100% and the impurity bands were subtracted to calculate the albumin purity. The percent of each contaminating protein in each band was calculated as the number of peptides identified for that contaminant protein as determined by peptide mapping divided by the total number of all peptides identified in a particular band. The image analysis was repeated 3 times such that the standard deviation is less than 0.5% out of 100%.

Determination of endotoxin by the Pyrogene rFC method.

Endotoxin content was determined by the Pyrogene rFC method. Lyophilized endotoxin standard was mixed with endotoxin free water as specified by the manufacturer (Lonza) to develop a standard curve. The protein samples were either diluted as is for liquid or alternatively, reconstituted with endotoxin free water for powder. Different dilutions were prepared such that the readings should appear within the range of the standard curve. The

samples were heated to 100 °C for 10 minutes to dissociate unwanted molecular interactions. In a typical experiment, the sample and standard were added at 100 µl per well, with 0, 0.001, 0.005, 0.01, 0.05, and 0.1 endotoxin units per well. The samples were also added at 100 µl and extra samples were included such that spiking with 0.001 -0.01 endotoxin units per well were added to test for assay inhibition or interference. The working reagent was prepared according to the manufacturer (Lonza) by mixing the rFC enzyme, assay buffer, and substrate in a 1:4:5 ratio, respectively. The working reagent was added to the wells at equal volume to the sample or standards. The fluorescence plate reader (Biotek FLX 800T) was set for excitation at 380 nm (bandwidth =20 nm) and the emission wavelength was set at 440 nm (bandwidth =30 nm). The reading taken at time zero is subtracted from the reading taken after 1 hour at 37 °C. The readings were considered valid if the correlation coefficient, slope, and Y-intercept for the standards was within the set limits, and the spiking experiments show that the spiked endotoxin was measureable and recoverable within the set limits. In addition, the standard deviation for duplicate samples should be in reasonable agreement such that the standard deviation was within a specified arbitrarily chosen limit. All samples were collected aseptically and the tubes/vials/containers used for testing were verified to be extremely low endotoxin following good laboratory practices as they relate to accurate and precise endotoxin testing.

Determination of Cell Viability

The hybridoma cell line AE1 (ATCC) was maintained in DMEM basic media containing 5% fetal bovine serum (FBS). Albumin was tested under serum-free conditions (AFM6, KC Bio, Kansas) without supplementation of fetal bovine serum. The cells were subculture from 5% FBS to serum free media over multiple passages. At each subculture, the cells were analyzed for total cell count and viability in the presence of the indicated concentrations of albumin. (As assessed by trypsinization and direct counting using a Neubauer haemocytometer). The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Date show the number of viable cells / ml divided by 10^5.

Determination of Detergent

The detergent concentration for the albumin was determined by a detergent (cell based) assay. Briefly, detergent sensitive cells were spiked with different amounts of detergent and the resulting cell viability cell determination used to generate a standard curve consisting of 16 independent data points. The change in viability with respect to the change in detergent

concentration was plotted and fitted with a logarithmic function. This equation was then used to calculate the unknown detergent concentrations in samples tested in the same cell based assay. The correlation coefficient for the standard curve for the data given was 0.9816. Typically detergent concentrations of greater than about 10 ppm per Cellastim dry weight, result in noticeable toxic activity. By comparison in a 10% albumin solution, toxic effects of detergent become apparent when the detergent concentration is above about 100 ppm to 200 ppm or 0.01% to 0.02 % (v/v).

Results & Discussion:

I. Analysis by Size Exclusion chromatography of plasma derived serum (Sigma Albumin), and recombinant HSA (Cellastim) produced using the new process (B000C) (Cellastim P0171) and the old process (Cellastim P0107).

The HPLC size exclusion profiles (**Figure 8A, C & D**) for the three types of albumin show that in terms of overall purity the different albumin preparations are generally similar. Specifically, the peaks at around 4.5 kDa and 240 kDa are the internal controls, while all three products contain a very small amount of an off main peak signal at about 10-12 kDa.

Human plasma derived albumin (Sigma Albumin) (**Figure 8A**), contains a contaminant at around 17 kDa, by comparison the recombinant rice derived albumin using the new process (Cellastim P0171) (**Figure 8C**) contains two protein contaminants of around 44 KDa and 55 kDa that occur in Cellastim made with the new process at significantly higher levels than when using the old process (Cellastim P0107) (**Figure 8B**). These peaks are not completely resolved in the HPLC separations, but can be seen as more clearly in the overlaid profiles of Cellastim P0171 and Sigma albumin (**Figure 8D**) and Cellastim made using the old and new processes shown in **Figures 8E.**

The proteins corresponding to these peaks represent about 5% of all of the contaminant proteins identified by Peptide Mass Fingerprinting analysis of the main albumin peak in Cellastim produced using the process described in Example 5, as discussed further below.

All albumin products tested also contained a peak at around 130 kDa that most likely represents albumin dimers, it is noticeable that the Cellastim dimer peak is significantly smaller than the plasma derived albumin. The creation of aggregated albumin is an indicator of protein

degradation which is used as one marker for degradation or loss of stability industry wide. It is likely that the Hsps present in Cellastim promote the disaggregation of the albumin, therefore reducing the number of dimers, since it is a commonly known function of Hsp 70 and other Hsp proteins.

II. Analysis by SDS PAGE of Cellastim batch **P0171** (New process) compared to albumin produced by Millipore/Novozymes (Cat No. 9301-01).

Results: SDS-PAGE analysis (**Figure 9A & B**) shows that in terms of overall purity the products are generally similar. **Figure 9A** shows a comparison of Cellastim P0171 and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **Figure 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

Visual inspection of the gel shows that the new process which meets more rigorous specifications is more consistent among the 3 lots tested. (**Figure 9B**, lane 2, 3, 4 vs. lane 6, 7, 8). The banding pattern is significantly different among the three samples from the previous process as compared to the new process. Importantly, the new process samples have significantly less aggregates at around 250 KDa than the old process samples have. (Average greater than 2 % for the old process, and average less than 1 % for the new process). The identity of the protein contaminates was that are enriched in Cellastim produced using the new process is discussed further below.

III. Analysis of endotoxin, detergent and growth promoting abilities of old and new batches of Cellastim. A comparison of the performance of the two different processes for preparing several different lots of albumin (**Tables E3 and E4**) demonstrates that the old process produced recombinant albumin that contained significantly more endotoxin, and detergent compared to the new process described in Example 5, and resulted in a product that significantly enhanced cell viability.

Table E3 Cellastim - Old Process B000								
Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					1mg/ml	2 mg/ml	5 mg / ml	10 /mg ml
2/27/08	B202	70.6	25.3	815.3	15	7.9	4.1	0.9
2/27/08	B203	58.8	23.1	267.7	15.8	12.4	6.5	4.1
2/28/08	B204	59.9	29.2	589.2	16.3	11.1	5.2	2.9
2/28/08	B205	63	64.3	133.4	14	11.6	6.9	3.8
3/3/08	B206	41.1	35.1	1182.2	14.9	2.3	0.0	0.0
3/3/08	B207	94.2	28.7	388	16.5	12.7	7.6	4
3/4/08	B208	24.5	>80	467.2	15.3	11.4	7.1	2.7
3/4/08	B209	66.1	91.8	406.4	16.7	15.2	6.9	4.1
3/4/08	B210	87.3	67.8	76.4	17.5	19.1	15.4	8.5
3/11/08	B217	62.09	4.4	127.3	16.7	18.7	13.2	6.6
Averages			**44.97**	**445.31**	**15.87**	**12.24**	**7.29**	**3.76**

Table E4 Cellastim - New Process B0000C								
Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					0.5mg/ml	2 mg/ml	5 mg / ml	10 /mg ml
2/2/2009	B0032C	209.4	0.35	3.1	13.1	15.7	13.4	11.2
2/2/2009	B0033C	271.4	0.26	7.9	18.4	17.4	15.1	14.3
2/10/2009	B0041C	247.1	0.18	5.4	15.4	17.2	14.1	12.1
4/27/2009	B0118C	617.4	0.11	Less than 1	15.7	15.4	19.2	16.8
5/14/2009	B0138C	610.6	0.48	Less than 1	12.5	15.8	15.1	14.6
6/9/2009	B0158C	598.0	0.13	3.9	13.7	17.1	13.2	11
6/15/2009	B0162C	618.6	0.20	2.0	15.5	17.5	17.2	14.7
7/28/2009	B0196C	507.0	0.36	3.9	17.4	17.3	18.8	14.8
8/26/2009	B0219C	851.4	0.86	8.2	17.9	16.3	16.6	13.7

8/31/2009	B0220C	897.8	0.93	Less than 1	13.5	16	15.5	15.1
9/9/2009	B0227C	929.9	0.13	Less than 1	11.1	14.4	12.5	11.4
Averages			**0.36**	**3.45**	**14.92**	**16.37**	**15.52**	**13.6**

Discussion: Re-engineering the old process to create the new process described in Example 5 resulted in significant changes in both overall product purity, and performance, as described more fully below.

The changes made it possible to make products that were lower in detergent, lower in endotoxin, and increased purity. Specifically, the new process routinely produced recombinant albumin with an overall purity of greater than about 95%. By comparison the old method routinely produced albumin with a maximum purity of about 90 %. Surprisingly, despite the increased product purity, these changes in processing also resulted in enhanced co-purification of heat shock proteins, (see below) with the recombinant albumin. Without being bound by any particular theory of operation, it is believed that the combination of high albumin purity, relative lack of endotoxin and / or detergent, and co-purification of heat shock proteins results in a product that significantly out performs previous methods for preparing albumin.

Specifically **Tables E3** and **E4** demonstrate that the new process for producing recombinant albumin results in a product that, for example at 5 mg /ml, results in an average batch to batch 100 percent improvement in cell viability (at 5 mg /ml), and also results in a product with an average 100-fold less endotoxin, and 100 fold less detergent than the old process.

Example 7: **Analysis of the effects on cell growth and viability.**

To compare the cell growth promoting abilities of the recombinant albumin prepared above, to other commercially available albumin products, the different sources of albumin they were compared side by side in a cell growth and viability assay. The three products tested were (Cellastim, Lot # P0153) Cellprime albumin (Millipore/Novozymes Cat No. #9301-01), and plasma derived albumin (Seracare Cat No. #HS-400-60).

Methods: Specially conditioned Hybridoma cells AE1 were seeded in DF12/ITSE at a density of 0.5 x 10^5 cells per ml of media after washing twice with same media to remove residual media.

The media and cells were then left untreated (negative control), treated with Seracare albumin, treated with Cellprime albumin, and treated with rice derived recombinant albumin at the concentrations shown in the figure legend. The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Results are shown in **Figure 10.**

Results: Novazyme's Cellprime albumin caused a loss in viability (cross-hatch bars). Seracare albumin (white bars) caused a measureable increase in viability but not as large an increase as is seen with recombinant albumin made in rice with the new process (Cellastim) (black bars). Under these conditions the rice derived recombinant albumin was approximately 5 times as active in promoting cell viability compared to any of the other albumin products, at any concentration tested. The negative control is represented by the striped bars.

Discussion: Given the possibility that the other commercially available albumin products may have similar overall purity, endotoxin and detergent levels to rice derived recombinant albumin, the dramatically superior performance of the recombinant rice derived albumin compared to other commercially available albumins suggests that the previously un-identified protein contaminants identified in Cellastim compared to the plasma derived albumin (Example 5) could be having a positive impact on cell viability. To identify and then characterize the impact of these proteins on the properties of Cellastim, a sample of the recombinant albumin was subjected to peptide mass finger printing, as described below.

Example 8: <u>Peptide Mass Finger printing of recombinant human serum albumin.</u>

Methods: Samples of albumin were analyzed to determine significant protein contaminants using a NanoLCMS/MS peptide sequencing system (ProtTech, Inc.), and proprietary software to identify the proteins based on the molecular weight of the peptide fragments. In brief, samples of albumin were analyzed by SDS-PAGE, and each major band gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin. The resulting peptide mixture was analyzed by a LC-MS/MS system, in which a high pressure liquid chromatography (HPLC) with a 75 micrometer inner diameter reverse phase C18 column was used in-line coupled with an ion trap mass spectrometer. The mass spectrometric data acquired was used to

search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting.

Results: Upon testing of three representative lots of recombinant albumin, three Hsp70 proteins were identified by Peptide Mass Fingerprinting (**Table E5**). The three specific sequences identified: ABF95267, ABA97211, and BAD 07938 were compared to the non redundant database to identify highly related and homologous proteins. The results of the top hits from each of these comparisons is shown in **Tables E6, E7 and E8** below.

Table E5

Peptides identified from Cellastim by mass finger printing

Sequence	Peptide
ABF95267	ATAGDTHLGGEDFDNRVVPGPADKSPMIVVTYKGEEK NAVITVPAYFN DSQRHNEPTAAAIAYGLDKK (**SEQ. ID. No. 2**)
AAB63469 BAD07938 BAD07713	NQAAVNPER NGHVFIIANDQGNRIVNKDGKPYIQVK IINEPTAAAIAYGLDKK KLGTVIGIDLGTTYSCVGVYK VEIESLFDGTDSFSEPLTR (**SEQ. ID. No. 3**)
ABA97211	NQADSVVYQTEK KQDTTTGASTLPKDEVERDVVLLDVTPLSLSLGLET LGGVMTK (**SEQ. ID. No. 4**)

Results of sequence comparisons to ABF95267 sequences in the non redundant database of protein sequences in Genbank® (Nucleic Acids Research, 2008 Jan;36(Database issue):D25-30) are shown in **Table E6** below:

Table E6

Sequences producing significant alignments with ABF95267:

Gene Refs	Gene description	(Bits)	Value
ref|NP_001140835.1|	hypothetical protein LOC100272911 [Zea ma...	79.7	8e-14
ref|XP_002465468.1|	hypothetical protein SORBIDRAFT_01g039390...	79.7	9e-14
ref|NP_001049719.1|	**Os03g0277300 [Oryza sativa (japonica cult...**	**79.7**	**9e-14**
gb|ACJ54890.1|	heat shock protein 70 [Oryza sativa Japonica G...	79.7	9e-14
Sp|P09189.1|	HSP7C_PETHY RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
emb|CAA31663.1|	hsp70 (AA 6 - 651) [Petunia x hybrida]	77.0	5e-13
ref|XP_002312089.1|	predicted protein [Populus trichocarpa] >...	77.0	5e-13
sp|P24629.1|	HSP71_SOLLC RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
gb|AAB99745.1|	HSP70 [Triticum aestivum]	76.6	6e-13
gb|AAB42159.1|	Hsc70 [Lycopersicon esculentum]	76.6	7e-13
gb|ACD45076.1|	heat-shock protein 70 [Dactylis glomerata]	76.3	8e-13
ref|XP_002512741.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
ref|XP_002512742.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
gb|AAA82975.1|	PsHSP71.2 >emb|CAA67867.1| heat shock protein ...	75.9	1e-12
gb|AAS09825.1|	heat shock cognate protein 70 [Thellungiella h...	75.9	1e-12
emb|CAA44820.1|	heat shock protein 70 [Nicotiana tabacum]	75.5	2e-12
ref|NP_001055754.1|	Os05g0460000 [Oryza sativa (japonica cult...	75.5	2e-12
ref|NP_001051724.1|	Os03g0821100 [Oryza sativa (japonica cult...	75.1	2e-12
ref|XP_002456611.1|	hypothetical protein SORBIDRAFT_03g039360...	75.1	2e-12
ref|NP_001044757.1|	Os01g0840100 [Oryza sativa (japonica cult...	75.1	2e-12
gb|ACR35910.1|	unknown [Zea mays]	75.1	2e-12
ref|XP_002284017.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002532297.1|	heat shock protein, putative [Ricinus com...	75.1	2e-12
ref|XP_002284008.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002283532.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.7	3e-12
ref|XP_002332067.1|	predicted protein [Populus trichocarpa	74.7	3e-12
gb|AAF34134.1|	high molecular weight heat shock protein [Malu...	74.7	3e-12
gb|EEC76425.1|	hypothetical protein Osl_14101 [Oryza sativa I...	74.7	3e-12
ref|XP_002316294.1|	predicted protein [Populus trichocarpa] >...	74.7	3e-12
ref|XP_002283516.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.3	3e-12
ref|XP_002441219.1|	hypothetical protein SORBIDRAFT_09g022580...	74.3	4e-12

Results of sequence comparisons of ABB63469 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E7** below:

Table E7

Sequences producing significant alignments with AAB63469:

Gene Refs	Gene description	(Bits)	Value
emb|CAP31983.1|	C. briggsae CBR-HSP-4 protein [Caenorhabditis...	50.1	7e-05
dbj|BAG60366.1|	unnamed protein product [Homo sapiens]	49.7	8e-05
ref|YP_002421952.1|	chapetone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|YP_001640420.1|	chaperone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|NP_001105893.1|	Binding protein homologl precursor [Zea m...	49.3	1e-04
gb|AAA62325.1|	HSP70	49.3	1e-04
ref|YP_001756576.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
gb|AAB63469.1|	endosperm lumenal binding protein [Oryza sativa]	49.3	1e-04
ref|YP_001925829.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
ref|NP_001105894.1|	Binding protein homolog2 precursor [Zea m...	49.3	1e-04
gb|ACF86491.1|	unknown [Zea mays]	49.3	1e-04
ref|NP_001045675.1|	**Os02g0115900 [Oryza sativa (japonica cult...**	49.3	1e-04
ref|ZP_02191025.1|	Molecular chaperone [alpha proteobacterium...	49.3	1e-04
ref|XP_001701685.1|	binding protein 1 [Chlamydomonas reinhard...	48.5	2e-04
ref|XP_001701884.1|	binding protein 2 [Chlamydomonas reinhard...	48.5	2e-04
emb|CAC37635.1|	luminal binding protein, BiP [Scherffelia dubia]	48.1	3e-04
gb|AAM93256.1|	heat shock protein 70-C [Heterodera glycines] ...	48.1	3e-04

Results of sequence comparisons of sequence ABA97211 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E8** below:

Table E8

Sequences producing significant alignments with ABA97211

Gene Refs	Gene description	(Bits)	Value
gb|AAK13022.1|	heat shock protein 70 [Fibrobacter succinogene...	44.3	0.004
ref|XP_002442079.1|	hypothetical protein SORBIDRAFT_08g009580...	44.3	0.004
gb|EEC69073.1|	**hypothetical protein OsI_37938 [Oryza sativa I...**	**44.3**	**0.004**
ref|XP_001752769.1|	predicted protein [Physcomitrella patens ...	44.3	0.004
ref|NP_001066486.1|	Os12g0244100 [Oryza sativa (japonica cult...	44.3	0.004
gb|ACT65562.1|	70 kDa heat shock protein [Triticum aestivum]	43.9	0.005
ref|NP_001152528.1|	stromal 70 kDa heat shock-related protein...	43.9	0.005
gb|ACN31310.1|	unknown [Zea mays]	43.9	0.005

ref|XP_001772650.1|	predicted protein [Physcomitrella patens ...	43.9	0.005
ref|NP_001146752.1|	hypothetical protein LOC100280354 [Zea ma...	43.9	0.005
gb|ABP65327.1|	chloroplast heat shock protein 70 [Pennisetum ...	43.9	0.005
gb|AAO72585.1|	heat shock-related protein [Oryza sativa (japo...	43.5	0.007
ref|YP_001740846.1|	Chaperone protein dnaK [Heat shock protei...	43.5	0.008
ref|ZP_03728467.1|	chaperone protein DnaK [Dethiobacter alkal...	43.1	0.009

Discussion: Peptide Mass Fingerprinting identified 3 rice heat shock protein super family members that co-purify with albumin, 2 Rice HSP70 genes, (gb|ACJ54890.1|), EEC69073, and AAB63469 - a BiP homolog from rice endosperm tissue (endosperm lumenal binding protein). The complete amino acid sequences coded by these genes are listed below:

Gene gb|ACJ54890.1| heat shock protein 70 [Oryza sativa Japonica Group] HSP70 was found to occur in recombinant albumin in Cellastim at approximately 0.07% wt / wt. Its complete amino acid coding sequence (**SEQ. ID. No. 5**) is provided below:

MAGNKGEGPA IGIDLGTTYS CVGVWQHDRV EIIANDQGNR TTPSYVAFTD TERLIGDAAK
NQVAMNPTNT VFDAKRLIGR RFSDPSVQAD MKMWPFKVVP GPADKPMIVV TYKGEEKKFS
121 AEEISSMVLT KMKEIAEAFL STTIKNAVIT VPAYFNDSQR QATKDACVIS GLNVMRIINE
181 PTAAAIAYGL DKKAASTGEK NVLIFDLGGG TFDVSILTIE EGIFEVKATA GDTHLGGEDF
241 DNRMVNHFVQ EFKRKHKKDI TGNPRALRRL RTACERAKRT LSSTAQTTIE IESLYEGIDF
301 YATITRARFE ELNMDLFRRC MEPVEKCLRD AKMDKAQIHD VVLVGGSTRI PKVQQLLQDF
361 FNGKELCKSI NPDEAVAYGA AVQAAILSGE GNQRVQDLLL LDVTPLSLGL ETAGGVMTVL
421 IPRNTTIPTK KEQVFSTYSD NQPGVLIQVY EGERTRTKDN NLLGKFELTG IPPAPRGVPQ
481 INVTFDIDAN GILNVSAEDK TTGKKNKITI TNDKGRLSKE EIERMVQEAE KYKAEDEQVR
541 HKVEARNALE NYAYNMRNTV RDEKIASKLP ADDKKKIEDA IEDAIKWLDG NQLAEADEFE
601 DKMKELESLC NPIISKMYQG GAGCPAGMDE DAPNGSAGTG GGSGAGPKIE EVD

AAB63469 BiP homolog from rice endosperm tissue (endosperm lumenal binding protein [Oryza sativa]) BiP was found to occur in recombinant albumin in Cellastim at about 0.09% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 6**) is provided below:

mdrvrgsafi lgvllagslf afsvakeetk klgtvigidl gttyscvgvy knghveiian
dqgnritpsw vafidserli geaaknqaav npertifdvk rdigrkfeek evqrdmklvp

```
121 ykivnkigkp yiqvkikdge nkvfspeevs amilgkmket aeaylgkkin davvtvpayf
181 ndaqrqatkd agviaglnva rlineptaaa laygldkkgg eknilvfdlg ggtfdvsilt
241 idngvfevla tngdthlgge dfdqrimeyf iklikkkysk diskdnralg klrreaerak
301 ralsnqhqvr veieslfdgt dfsepltrar feelnndlfr ktmgpvkkam ddagleksqi
361 heivlvggst ripkvqqllr dyfegkepnk gvnpdeavay gaavqqgvls geggdetkdi
421 lildvaplsl gietvggvmt klipmntvip tkksqvftty qdqqttvsiq vfegersmtk
481 dcrligkfdl sgipaaprgt pqievtfevd angilnvkae dkgtgkseki titnekgrls
541 qeeidrmvre aeefaeedkk vkeridarnq letyvynmkn tvgdkdklad kleseekekv
601 eealkealew ldenqtaeke eyeeklkeve avcnplisav yqrtggapgg rrrgrlddeh
661 del
```

EEC69073 / Osl_37938 [Oryza sativa Indica Group] The stromal HSP70 was found to occur in recombinant albumin in Cellastim at about 0.06% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 7**) is provided below:

```
masftsqlga macgaapsts plaarrsgql fvgrkpaaas vqmrvpragr argvamrvac
61 ekvvgidlgt tnsavaameg gkptvitnae ggrttpsvva ytkggerlvg qiakrqavvn
121 pentffsvkr figrkmaevd deakqvsyhv vrddngnvkl dcpaigkqfa aeeisaqvlr
181 klvddaskfl ndkitkavvt vpayfndsqr tatkdagria glevlriine ptaaslaygf
241 ekknnetilv fdlgggtfdv svlevgdgvf evlstsgdth lggddfdkfy fcwvfyfgam
301 thetpkvvdw lasnfkkdeg idllkdkqal qrlteaaeka kmelstlsqt nislpfitat
361 adgpkhiett lsrakfeelc sdllidrlktp vtnalrdakl svdnldevil vggstripsv
421 qelvkkltgk dpnvtvnpde vvslgaavqg gvlagdvkdv vlldvtplsl gletlggvmt
481 klipmnttlp tsksevfsta adgqtsvein vlqgerefvr dnkslgsfrl dgippaprgv
541 pqievkfdid angilsvaai dkgtgkkqdi titgastlpk devermveea dkfaqedkek
601 rdaidtknqa dsvvyqtekq lkelgdkvpa pvkekvdakl nelkeaiagg stqsmkdama
661 alneevmqig qamynqqpna gaagptpgad agptssggkg pndgdvidad ftdsn
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Because these proteins only occur at low levels in the new batches of rice recombinant albumin relative to albumin, a pre-requisite for confirming that these contaminants are actually responsible for the superior growth promoting effects of the new batches of recombinant albumin is to determine whether the addition back of these components to albumin restores or enhances the growth promoting activities of the recombinant albumin at levels which are comparable to those actually identified for each component in Cellastim.

Example 9: Separation of heat shock proteins from recombinant albumin by affinity chromatography.

Methods: Cellastim produced using the new process [Lots P0153, P0156, and or P0171] powder was mixed with purified water at approximately 20 g/L. The resulting solution was diafiltered against 50 mM Tris/Cl, pH 7.0 with at least 5 equal volumes of buffer. The resulting solution was passed over an ATP agarose column and the resulting flow through was labeled as fraction A. The column was washed with 5 column volumes of the equilibration buffer and the material bound to the ATP-agarose was eluted with 50 mM Tris/Cl, 1M KCl, pH7.0. The eluted material was labeled as fraction B. The wash was kept as fraction C. Fraction A was directly concentrated to 100 g/L and diafiltered with d-PBS. Fraction B was concentrated significantly, up to 20 fold or 100 fold in 50 mM Tris/Cl for further analysis. The wash fraction C was kept for further reference. For Western blotting, 10 µg of each protein fraction (by A280, where the e.c. (extinction coefficient) of albumin is 0.53 cm^2/mg and e.c. of Hsp70 is 0.41 cm^2/mg) were loaded on a 4-20% SDS PAGE gel in 2X SDS loading buffer. The samples were heated to 80 °C for approximately 5 minutes before loading. The separation was done at 200V (constant voltage) and ran for approximately 90 minutes. The resulting gel was rinsed in water for 30 minutes to 2 hours and then the proteins were transferred to a Nitrocellulose membrane at 30 mA (constant current) for 2 hours. The resulting blot contained the molecular weight marker proteins as a transfer control and was then blocked in 5% (w/v) milk powder in water. The primary monoclonal antibody (a mouse anti-bovine Hsp70(Sigma/Aldrich #H5147)) was added in 5% milk solution to the blot (1:2500) and the blot was incubated on a rocker with gentle rocking overnight at 4 °C. The blot was then washed 4 times for 10 minutes each in TDN and the secondary antibody (Pierce anti-mouse HRP conjugated) in 5% milk solution which was added at a dilution of 1:2500. After incubation at 4 °C for 2 to 3 hours, the blot was washed 4 times with TDN for 10 minutes each. The resulting blot was then incubated with pico (Pierce) chemiluminescent substrate for 5 minutes. Kodak photographic film was exposed to the blot in a dark room and the subsequent film was developed, rinsed, fixed, rinsed, and dried. To determine accurate transfer of the molecular weight marker position onto the film, a light emitting label was used.

Results: The results are shown in **Figure 11.** The Western blot pictured shows that the separation scheme produces two populations of proteins in the A (flow through) and B (ATP binding) fractions. The starting material, (lane 2) the fraction A flow through, (lane 3) fraction C wash, (lane 4) and fraction B (lane 5) were tested for the ability to react to the monoclonal antibody. In addition, a commercially available Hsp70 protein that serves as a positive control

was loaded in the last lane (lane 10). As shown in the blot in **Figure 11**, the flow through fraction A (lane 3) does not contain significant amounts of Hsp70. The eluted and concentrated fraction B (lane 4) is highly reactive to the antibody as shown in the blot and indicates at least two distinct bands centered around the 75 kDa molecular weight marker. The wash fraction C (lane 5), indicates the presence of two bands that run at slightly below 75 kDa. In a separate independent experiment, the flow through fraction A (lane 7) again is not reactive to the antibody, and the wash fraction C (lane 8) is also not reactive to the antibody, but the fraction enriched in ATP binding proteins (Fraction B) shown in lane 9 gives the same banding pattern as was seen from the first separation.

Discussion: A separation protocol was developed to separate HSP70 proteins based on their ability to bind to ATP agarose affinity resin, and, was tested for its effectiveness. The procedure involved only minimal sample manipulation, using only ATP agarose, and ultrafiltration to concentrate and conduct buffer changes. and an anti-hsp70 antibody to detect the presence of hsps. The results of the procedure (**Figure 11**) clearly demonstrates that while in 10 µg of starting material, 10 µg of flow through, or 10 µg of wash fraction there is insufficient hsp70 to be detected by the ant-Hsp antibody. By contrast, the fraction eluted from the ATP agarose column, contains at least two proteins that are clearly recognized by the anti-Hsp70 antibody. The results therefore show that the separation scheme was successful and reproducible on two independent chromatography runs and diafiltrations. Furthermore. the data substantiates the identification of heat shock proteins made by Peptide Mass Finger printing, as discussed earlier, and demonstrates that these proteins are functional and can be readily isolated and enriched by simple ATP agarose chromatography followed with diafiltration. It is concluded that the heat shock proteins co-purify with the recombinant albumin. Such co-purification is consistent with the hypothesis that the heat shock proteins are bound to the albumin, and that the albumin acts to stabilize the heat shock proteins in a stable conformation. Surprisingly, the recombinant albumin / heat shock protein complex retains significant ATPase activity (data not shown) consistent with the presence of function heat shock proteins. This increased activity was further confirmed as providing a growth promoting effect as described below.

Example 10: <u>Impact of the removal of Hsps from rice recombinant albumin on cell viability.</u>

Methods: The separation scheme described in Example 9 was also used to produce fraction A suitable for cell culture testing (**Figure 12**). The method involves minimal manipulation of fraction A, as it is flowed through an ATP agarose column and then concentrated by diafiltration and buffered with PBS that is suitable for cell culture. The intent of the method is to not

introduce new variables into the experiment such that a loss of viability is seen but due to some other reason or cause beyond the removal of ATP binding proteins. Fraction A was tested against the unadulterated control (starting material) for ability to promote hybridoma cell culture viability. The results of the test are shown in **Figure 12**.

Results: As shown in **Figure 12** the Cellastim starting material (cross hatched bars), and Part A (solid bars) were tested at the same concentration and compared to the negative control (striped bars). A statistically significant decrease is observable at all four concentrations tested. The result indicates that there was a significant loss in the performance of Cellastim after ATP agarose treatment. The treatment resulted in a 28.0, 21.7, 26.7, and 79.5 % loss as compared to Cellastim before removal of ATP binding proteins. In this experiment, care was taken in the design and handling of the samples to ensure that any inadvertent losses in performance due to sample handling or the accidental introduction of new contaminants were minimized.

Discussion: The cell culture results (**Figure 12**) demonstrate that it is possible to reduce the performance of the rice derived recombinant albumin by simply passing it over an ATP binding column. This data, when combined with the results shown in Example 9 demonstrates that the depletion of the hsps from albumin by the ATP agarose column directly reduces the cell growth promoting properties of the albumin. This result therefore demonstrates that the superior properties of the albumin arise, at least in part, from the contaminating heat shock proteins in the albumin.

Example 11: <u>Effect of rice derived recombinant human serum albumin in combination with lactoferrin on the proliferation of CHO K1 in serum free culture.</u>

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components when added in combination with these components, a series of additional experiments were designed to evaluate and identify optimum combinations of such components.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200

nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or rLF or various mixtures of the two additives. Control cultures without added rHSA or rLF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. All cultures were grown at 37°C, 6% CO_2, in a humidified environment. The Observed Effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Table E9 shows the number of viable cells after 6 days of culture. Cells in media without supplementation proliferated to 3.3 x 10^5 viable cells/ml. Addition of plant derived recombinant human serum albumin (rHSA) or recombinant lactoferrin (rLF), or a combination of the two increased the proliferation of cells as indicated below. Column two shows the Observed Effect of the respective additive(s) above baseline control culture. Column 3 shows the Predicted Effect using a model where the predictive additive effect equals the sum of additive1 and additive2.

TABLE E9

	Viable cells(e^5)/ml	Observed Effect	Predicted Effect	Ratio of Lactoferrin to albumin
No Additives (Baseline)	3.3	0		
rHSA 0.5 g/L	7.3	4.0		
rHSA 1 g/L	7.9	4.6		
rLF 0.1 g/L	7.5	4.2		
rLF 0.8 g/L	16.0	12.7		
rLF 0.1 g/L +rHSA 0.5 g/L	18 9	15.6	8.2	1 : 5
rLF 0.8 g/L +rHSA 0.5 g/L	29.0	25.7	16.7	1 : 0.625
rLF 0.1g/L +rHSA 1g/L	23.6	20.3	8.8	1 : 10
rLF 0.8g/L +rHSA 1g/L	35.4	**32.1**	17.3	: 1.25

Discussion: Lactoferrin is an essential factor for serum free cell culture media, where it acts to transport iron; additionally Lactoferrin has more recently been identified as a growth factor and inhibitor of apoptosis in some cell types (Huang et al., In vitro Cell. Dev. Biol. Anim. (2008) 44(10) 464-71; Wong et al., Rheumatology 48(1) 39-44; Bi et al., Arch. Immunol. Ther. Exp. (1997)45(4) 315-20;Hashizume et al., Biochem. Biophys. Acta. (1983) 763(4) 377-82). In **Table E9**, the data shows that the addition of 0.1 g/L and 0.8 g/L recombinant lactoferrin resulted in a significant enhancement of cell growth compared to the baseline rate of cell growth consistent with lactoferrins growth stimulating properties.

The growth stimulating effect of lactoferrin was further enhanced by the addition of plant derived recombinant human serum albumin. Unexpectedly the combination of 0.1 g/L lactoferrin and 0.5 g/L plant derived recombinant human serum albumin (1 to five ratio) resulted in a stimulation in cell growth that was approximately 4 fold higher than either albumin alone or lactoferrin alone tested at the same concentrations. While the combination of 0.1 g/L lactoferrin and 1.0 g/L human serum albumin (1 to 10 ratio) resulted in a stimulation in cell growth that was approximately 4.4 to almost 5 fold higher than either albumin or lactoferrin alone at the same concentrations.

Synergism was also observed when plant derived recombinant human serum albumin was added to the lactoferrin at a concentration of 0.8 g/L. Even at this significantly higher concentration, the addition of plant derived recombinant human serum albumin further increased cell growth by 2 to 2.5 fold.

Albumin and lactoferrin are distinct protein families with little overlapping structural or functional similarity. Thus the fact the synergism was observed with these proteins is unexpected based on their dissimilar structure & function. Synergism provides the opportunity to develop improved supplements that provide for supplements with enhanced cell culturing properties (i.e. improved cell growth and viability) by using optimal ratios of lactoferrin and albumin.

Example 12: <u>Effect of rice derived recombinant albumin in combination with lactoferrin on the productivity of CHO K1 cells grown in serum free culture.</u>

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL

penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of plant derived recombinant human serum albumin or LF or various mixtures of the two additives. Control cultures without added HSA or LF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. Antibody productivity was determined at the end of the batch culture when cell viability had declined to <10% (typically day 13 or 14) by quantitative ELISA (Bethyl Laboratories). All cultures were grown at 37°C, 6% CO_2, in a humidified environment. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

TABLE E10

	Product Conc. (µg/ml)	Observed Effect	Predicted Effect	Ratio of HSA to Lactoferrin
No Additives (Baseline)	47.1	0		
rHSA 0.5g/L	114.6	67.5		
rHSA 1g/L	136.1	89.0		
rLF 0.1g/L	91.9	44.8		
rLF 0.8 g/L	138.5	91.4		
rLF 0.1g/L + rHSA 0.5g/L	183.7	136.6	112.3	1 : 5
rLF 0.8g/L + rHSA 0.5g/L	242.1	195.0	158.9	1 : 0.625
rLF 0.1g/L + rHSA 1g/L	219.3	172.2	133.8	1 : 10
rLF 0.8g/L + rHSA 1g/L	260.2	213.1	180.4	1: 1.25

Table E10 shows the effect of plant derived recombinant human serum albumin and recombinant lactoferrin produced in monocot cells, and various combinations of the two proteins on the productivity of CHO K1 cells. Table E10 shows that the concentration of antibody product produced by the cells after 13 days of culture in the baseline medium (with no additives) produced 47.1 µg/ml of antibody product. Medium supplemented with recombinant albumin or recombinant lactoferrin alone or a combination of plant derived recombinant human serum albumin and lactoferrin produced significantly higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the predictive additive effect of the combination of two additives using a model where the predictive additive equals the sum of each additive alone at the same concentration.

Discussion: Table E10 demonstrates that improvements in cell growth and viability directly translated into improved antibody expression and cell productivity. As was the case for cell viability and cell growth, maximal effects of the protein components was observed when these were added to the culture together.

Table E11 shows an additional data set showing the effect of the additives at an expanded range of concentrations, using the same conditions as in **Example12.** Medium supplemented with recombinant albumin alone, or lactoferrin alone, or a combination of both proteins, produced higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the Predicted Effect of the combination of two additives using a model where the Predicted Effect equals the sum of the effects of each additive when added alone at the same concentration. The data indicates that the actual effect of the combination is greater than the Predicted Effect over all of the ratios of the two proteins tested.

TABLE E11					
	Product Conc. (ug/ml)	**Observed Effect**	**Predicted Effect**	**% Improvement over predicted activity**	**Ratio of Lactoferrin to HSA**
No additives (Baseline)	56.9	0.0			
Single additives					
HSA 0.5g/L	99.1	42.2			
HSA 1 g/L	126.3	69.4			
HSA 2 g/L	186.0	129.1			
HSA 3 g/L	212.9	156.0			
LF 0.1g/L	123.1	66.2			
LF 0.5g/L	173.5	116.6			
LF 0.8g/L	182.0	125.1			
LF 1.5g/L	196.3	139.4			
Combinations					
LF 0.1g/L+ HSA 0.5g/L	212.4	155.5	108.5	143%	1 : 5
LF 0.5g/L+ HSA 0.5g/L	314.9	258.0	158.9	163%	1: 1
LF 0.8g/L+ HSA 0.5g/L	302.8	245.9	167.4	147%	1: 0.625
LF 1.5g/L+ HSA 0.5g/L	336.8	279.9	181.7	154%	1: 0.33
LF 0.1g/L+ HSA 1.0g/L	239.7	182.8	135.7	133	1: 10
LF 0.5g/L+ HSA 1.0g/L	339.4	282.5	186.1	152	1: 2
LF 0.8g/L+ HSA 1.0g/L	357.7	300.8	194.6	155	1: 1.2
LF 1.5g/L+ HSA 1.0g/L	343.7	286.8	208.9	137	1: 0.66
LF 0.1g/L+ HSA 2.0g/L	261.3	204.4	195.4	105	1: 20

LF 0.5g/L+ HSA 2.0g/L	377.2	320.3	245.8	131	1: 4
LF 0.8g/L+ HSA 2.0g/L	414.8	357.9	254.3	141	1: 2.4
LF 1.5g/L+ HSA 2.0g/L	358.0	301.1	268.6	112	1: 1.33

Discussion: Ratios of lactoferrin to albumin as low as 1 to 0.333 to as high as 1: 20 produced synergistic stimulations of cell growth. Optimal ratios of lactoferrin to albumin were found at 1 to 0.333 wt/wt (at an albumin concentration of 0.5 g/L); at 1 to 1.2 wt/wt (at an albumin concentration of 1.0 g/L); and at 1: 2.4 wt/wt (at an albumin concentration of 2.0 g/L).

Thus the stimulation of cell productivity occurs over a broad range of ratios of recombinant albumin and lactoferrin. Optimal ratios of lactoferrin and recombinant albumin vary to a certain extend on the absolute concentration of both protein components. Preferred ratios of Lactoferrin to Albumin range from about 1 (lactoferrin) to 0.33(albumin) to about 1 (lactoferrin) to 3 (albumin) wt/wt.

Example 13: <u>Evaluation of the effect of combinations of albumin and transferrin in the presence of other cell culture components.</u>

To determine the effect of other cell culture components on the synergistic effect of lactoferrin and albumin on cell growth and viability, a series of additional experiments were carried out with other cell culture components which have been previously shown to be essential for cell growth in serum free conditions.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments transferrin (TF) was used at an initial concentration of 5.5 µg/mL; Plant derived recombinant Human Serum Albumin (HSA) was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite (SE) and Ethanolamine were used at concentrations of 1:500,000 v/v and 6.7 µg/mL respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the supplements listed above, as described in **Table E12** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E12

	Viable cells/ ml	Std	Observed Effect	Predicted Effect	Synergistic	Ratio of transferrin to albumin
DMEM/F12 alone	0.01	0.01				
10% FBS	11.40	0.71	11.39			
TF 5.5 µg/L	0.02	0.00	0.01			
SE 6.7 µg/L	0.22	0.13	0.21			
HSA 0.1 g/L	2.31	0.06	2.30			
HSA 0.5 g/L	4.67	0.18	4.66			
HSA 1 g/L	4.55	0.46	4.54			
doubles						
TF+SE	0.40	0.04	0.38	0.21	yes	
TF+ HSA 0.1 g/L	3.00	0.02	2.98	2.30	yes	1:18
TF+ HSA 0.5 g/l	5.42	0.20	5.41	4.67	yes	1:90
TF+ HSA 1 g/L	5.71	0.14	5.69	4.55	yes	1:181
doubles						
SE+ HSA 0.1 g/l	2.76	0.06	2.75	2.50	Yes/no	
SE+ HSA 0.5 g/L	4.15	0.16	4.14	4.87	no	
SE+ HSA 1 g/L	3.75	0.22	3.74	4.75	no	
Triples						

TF+SE+ HSA 0.1 g/L	7.40	0.00	7.39	2.51	yes	*1:18*
TF+SE+ HSA 0.5 g/L	9.48	0.31	9.46	4.87	yes	*1:90*
TF+SE+ HSA 1 g/L	9.71	0.11	9.70	4.75	yes	*1:181*

Discussion: Recombinant human serum albumin in combination with recombinant transferrin showed synergistic effects when added to culture media together. This effect was further augmented in the presence of selenium. However the addition of selenium did not act synergistically with recombinant plant derived human serum albumin alone. Maximal synergistic effects of transferrin and plant derived recombinant human serum albumin occurred in the presence of selenium and at ratios of transferrin to plant derived recombinant human serum albumin of about 1 (transferrin) to 100 (albumin) wt/wt, and of about 1 (transferrin) to 200 (albumin).

Selenium is a well known essential trace element for cell growth and development, and its positive role in biological systems includes detoxification of free radicals by activating glutathione peroxidase. Additionally selenium can act as a highly effective iron carrier in serum free media, which helps to explain its synergistic impact on transferrin stimulated cell growth. (Zhang et al., Biotechnol. Bioeng. (2006) 95(6) 1188-97).

Example 14: <u>Evaluation of the effect of combinations of albumin, transferrin and insulin.</u>

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin, plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination with insulin was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 0.1 g/L, 0.5 g/L, and 1 g/L & Sodium Selenite and Ethanolamine were used at concentrations of 6.7 µg/mL and 1:500,000 v/v and respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E13** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37ºC, 6% CO_2, in a humidified environment, and shaken with 2 cm rotational orbit at 120 RPM.

TABLE E13

	Viable cells/ml	Std	Observed Effect	Predicted Effect	Synergistic
D/F12 alone	0.01	0.01			
10% FBS	11.65	0.21	11.64		
Factors Alone					
Insulin (IN) 10 µg/L	0.04	0.02	0.03		
Transferrin (TF) 5.5 µg/L	0.02	0.00	0.01		
Sodium Selenite (SE) 6.7 µg/L	0.22	0.13	0.21		
HSA 0.1 g/L	2.31	0.06	2.30		
HSA 0.5 g/L	4.67	0.18	4.66		
HSA 1 g/L	4.55	0.46	4.54		
Doubles					
IN+TF	0.03	0.02	0.02	0.06	
IN+SE	1.06	0.15	1.05	0.26	Yes
IN+ HSA 0.1 g/L	2.54	0.08	2.53	2.35	No
IN+ HSA 0.5 g/L	3.96	0.34	3.95	4.71	No
IN+ HSA 1 g/L	4.61	0.16	4.60	4.59	No
Triples					
IN+TF+SE	2.13	0.49	2.12	0.28	Yes
IN+TF+ HSA 0.1 g/L	3.29	0.03	3.28	2.37	Yes
IN+TF+ HSA 0.5 g/L	5.84	0.49	5.83	4.73	Yes
IN+TF+ HSA 1 g/L	6.84	0.34	6.83	4.61	Yes

Triples					
IN+SE+ HSA 0.1 g/L	2.21	0.17	2.19	2.37	No
IN+SE+ HSA 0.5 g/L	3.41	0.21	3.39	4.73	No
IN+SE+ HSA 1 g/L	3.62	0.23	3.60	4.61	No
Quaternaries					
IN+TF+SE+ HSA 0.1 g/L	7.91	0.46	7.90	2.59	Yes
IN+TF+SE+ HSA 0.5 g/L	9.14	0.63	9.13	4.95	Yes
IN+TF+SE+ HSA 1 g/L	10.07	0.67	10.05	4.83	Yes

Discussion: In these studies the addition of insulin and plant derived recombinant human serum albumin did not produce a synergistic effect on cell growth. By contrast the addition of plant derived recombinant human serum albumin and transferrin and insulin did produce a synergistic effect on cell growth.

Example 15: Evaluation of the effect of combinations of albumin, transferrin and insulin in the presence of Selenium.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin,(HSA) insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 6.7 µg/m L Sodium Selenite at 0.4x 10^5 viable cells/mL. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL Ethanolamine were used at a concentration of 1:500,000 v/v. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E14** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of

viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37ºC, 6% CO_2, in a humidified environment.

TABLE E14

Condition	Viable cell count 10e5	Std	Observed Effect	Predicted Effect	% Improvement over predicted activity	Synergistic
Base medium of D/F12+ Selenium	0.08	0.04	0.00			
10% FBS	13.20	0.28	13.12			
Insulin	0.30	0.09	0.22			
Factors Alone						
TF	0.27	0.04	0.19			
HSA 0.1 g/L	2.23	0.11	2.15			
HSA 0.5 g/L	6.21	0.20	6.13			
HSA 1 g/L	8.00	0.05	7.91			
doubles						
IN+TF	0.48	0.02	0.40	0.41		No
IN+ HSA 0.1 g/L	1.81	0.02	1.72	2.37		No
IN+ HSA 0.5 g/L	6.00	0.09	5.91	6.35		No
IN+ HSA 1 g/L	7.43	0.19	7.35	8.13		No
doubles						
TF+ HSA 0.1 g/L	4.29	0.04	4.20	2.34	179	Yes
TF+ HSA 0.5 g/L	12.35	0.21	12.27	6.32	194	Yes
TF+ HSA 1 g/L	15.48	0.71	15.39	8.1	190	Yes
Triples						
IN+TF+ HSA 0.1 g/L	4.18	0.21	4.09	2.56	159	Yes

IN+TF+ HSA 0.5 g/L	13.60	0.00	13.52	6.54	206	Yes
IN+TF+ HSA 1 g/L	15.38	0.22	15.29	8.32	184	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin.

Example 16: Evaluation of the effect of combinations of albumin and transferrin in the presence of Ethanolamine.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin (HSA), insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 1:500,000 Ethanolamine v/v at 0.4x 10^5 viable cells/ml. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL. transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite was used at a concentration of 6.7 µg/mL. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E15** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37ºC, 6% CO_2, in a humidified environment.

TABLE E15				
Condition	**Observed Effect (Viable cells/ml)**	**Std**	**Predicted Effect (Viable cells/ml)**	**Synergistic**
Base Medium of D/F12+ Ethanolamine	0.51	0.16		
10% FBS	14.45	0.64		
IN	1.41	0.24		
Factors Alone				
TF	2.08	0.15		
HSA 0.1 g/L	2.16	0.07		
HSA 0.5 g/L	5.66	0.35		
HSA 1 g/L	7.08	0.21		
doubles				
IN+TF	3.12	0.11	3.48	No
IN+ HSA 0.1 g/L	1.87	0.00	3.57	No
IN+ HSA 0.5 g/L	4.48	0.25	7.07	No
IN+ HSA 1 g/L	5.14	0.19	8.49	No
doubles				
TF+ HSA 0.1 g/L	6.72	0.35	4.24	Yes
TF+ HSA 0.5 g/L	12.30	0.26	7.74	Yes
TF+ HSA 1 g/L	16.20	0.65	9.16	Yes
Triples				
IN+TF+ HSA 0.1 g/L	7.46	0.28	5.64	Yes
IN+TF+ HSA 0.5	13.30	0.36	9.15	Yes

g/L				
IN+TF+ HSA 1 g/L	16.60	0.48	10.57	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin, over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin under these conditions.

Surprisingly the combination of Insulin, transferrin and plant derived recombinant human serum albumin produced superior cell growth compared to 10 % serum, demonstrating the superior properties of these combinations of proteins.

Example 17: <u>Comparison of rice recombinantly produced albumin and plasma derived albumin.</u>

To directly compare the effect of rice derived recombinant human serum albumin (rHSA) to plasma derived serum albumin (pHSA), samples of both were directly compared at two ratios when mixed with rice derived recombinant transferrin for their ability to stimulate cell growth.

Methods: Hybridoma cells were grown in DMEM/F12 (Gibco) in 10% FBS. Cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in either 1)DMEM/F12 base medium, 2) DMEM/F12+sodium selenate base medium or 3) DMEM/F12 base medium at $0.75x10^5$ viable cells/ml. Each base medium was further supplemented with either recombinant transferrin (TF), rice derived recombinant human serum albumin (rHSA), plasma derived human serum albumin (pHSA) or combinations of both transferrin and either form of albumin. After 3 days of incubation at 37° C, 5% CO_2, the concentration of viable cells was determined by a Guava PCA cell counter as instructed by the manufacturer. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components. The Effect Factor was calculated as the observed effect divided by the expected effect. Effect factor of 1 is indicative of expected effect (additive). Effect factor >1 is indicative of more than additive or synergistic effect.

In these experiments transferrin (TF) was used at an initial concentrations of 1 and 3 mg/L; albumins were used at concentrations of 5 g/L, Sodium Selenite was used at a concentration of 6.7 µg/L & Ethanolamine was used at 1:500,000 v/v.

5

Table E16

Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual / expected
Base Medium	0.11	0.07			
Components alone					
rTF 1 mg/L	0.21	0.01	0.10		
rTF 3 mg/L	0.28	0.09	0.18		
pHSA 5 g/L (Seracare)	11.30	0.28	11.19		
rHSA 5 g/L (Cellastim)	5.11	0.02	5.00		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	12.85	0.49	12.74	11.51	1.1
rTF3 mg/L + pHSA 5 g/L	13.20	0.42	13.09	16.41	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	18.80	0.71	18.69	5.31	3.5
rTF3 mg/L + rHSA 5 g/L	18.85	0.92	18.74	5.39	3.5

Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual/expected
Base Medium	0.04	0.02			

Components alone					
rTF 1 mg/L	0.14	0.01	0.10		
rTF 3 mg/L	0.14	0.05	0.10		
pHSA 5 g/L	6.08	0.13	6.04		
rHSA 5 g/L	1.76	0.17	1.72		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	7.46	0.49	7.42	6.22	1.2
rTF3 mg/L + pHSA 5 g/L	7.84	0.05	7.79	7.84	1.0
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	8.55	0.79	8.51	1.90	4.5
rTF3 mg/L + rHSA 5 g/L	8.37	0.23	8.33	1.90	4.4

Base Medium:DMEM/F12	**Viable cells/ml 10e5**	**Std**	**Observed Effect**	**Predicted Effect**	**Effect Factor (actual/ expected**
Base Medium	1.01	0.02			
Components alone					
rTF 1 mg/L	3.45	0.27	2.44		
rTF 3 mg/L	3.47	0.27	2.46		
pHSA 5 g/L	13.15	0.49	12.14		
rHSA 5 g/L	3.46	0.13	2.45		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	14.83	0.49	13.82	16.60	0.8
rTF3 mg/L + pHSA 5 g/L	14.57	0.12	13.56	16.61	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	17.87	1.46	16.86	6.91	2.4

rTF3 mg/L + rHSA 5 g/L	20.20	1.13	19.19	6.93	2.8

Discussion: Table E14 shows the results of three independent experiments that confirm that the combinations of TF with rice derived recombinant human serum albumin produced Observed Effect values much greater than the Predicted Effect. By comparison, the combination of ***plasma***-derived HSA and transferrin produced values that were similar to, or less than, the expected effect. Thus the ability of albumin to synergistically enhance the activity of transferrin and lactoferrin appears to be dependent on the source of albumin, rather than being an inherent characteristic of the albumin itself. Specifically the present experiments establish that the synergistic properties of human serum albumin with transferrin related proteins such as transferrin and lactoferrin is an unexpected and surprising property of rice derived recombinant human serum albumin. Significantly the combination of transferrin and plant derived recombinant albumin was more effective in stimulating cell growth than the combination of plasma derived albumin and transferrin, even though plasma derived albumin was more effective at 5 g/L alone than the same concentration of rice derived human serum albumin.

Example 18: <u>Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with either plasma derived or recombinant transferrin.</u>

To directly compare the effect of rice derived recombinant human transferrin (rTF) to plasma derived transferrin (pTF), samples of both were directly compared when mixed with rice derived recombinant human serum albumin for their ability to stimulate cell growth.

Methods: A base medium (BM) was composed that consisted of DMEM/F12+ with Sodium Selenite 0.0067 mg/L, Ethanolamine 1:500,000 v/v. Base medium included human plasma transferrin, pTF at a concentration of 5.5 mg/L in combination with either plasma HSA (pHSA, cell culture grade Seracare) or rice derived recombinant HSA (rHSA). Hybridoma cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in at 0.80×10^5 viable cells/ml and incubated at 37° C, 6% CO_2. The concentration of viable cells was determined daily by a Guava PCA cell counter as instructed by the manufacturer. The concentration of antibody produced by the cells and secreted into the medium was determined on day 6 by quantitative ELISA (Bethyl laboratories). Error bars indicate standard deviation. The production of antibody with the combination of pTF +rHSA was similar to that of DMEM/F12+10% FBS.

Discussion: Figure 13 shows that rice derived recombinant human serum albumin was able to act synergistically with plasma derived transferrin to significantly enhance the growth and productivity of the cells. By comparison, plasma derived serum albumin was unable to exert a similar stimulatory effect. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective with transferrin related proteins that were either plasma derived, or recombinant.

Example 19: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with insulin related growth factor-1.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components such as the growth factor IGF-1, additional experiments were performed to assess this possibility.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) as described previously. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and recombinant insulin related growth factor 1 (IGF1) (Ajinomoto) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate. 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or IGF-1 or various mixtures of the two additives. Control cultures without added rHSA or IGF-1 were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. All cultures were grown at 37ºC, 6% CO_2, in a humidified environment. Table E17 shows the results of the experiment. Here the Observed Effect was calculated as the net viable cell concentration (observed-base medium), and the Predictive Effect was calculated as the sum of the effect of the individual components.

Table E17

	viable cells/ml 10e5	Observed Effect (Cell count-baseline)	Predicted Effect
No Additive(baseline)	3.3	0.0	
HSA 0.5 g/L	7.3	4.0	
HSA 1g/L	7.9	4.6	
IGF-1 30 nM	12.0	8.7	
HSA 0.5 g/L+ IGF-1 30nM	23.5	15.6	12.7
HSA 1 g/L+ IGF-1 30nM	25.6	13.6	13.3

Results and Discussion: **Table E17** shows that rice derived recombinant human serum albumin was able to act synergistically with recombinant IGF-1 to significantly enhance the growth and productivity of the cells. By comparison, recombinant albumin did not act synergistically with insulin (Table E15) suggesting that synergism is not a general effect across all cell culture components. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective in combination with IGF-1.

It will, of course, be appreciated that the above description has been given by way of example only and that modifications in detail may be made within the scope of the present invention.

The invention is capable of considerable modification, alteration, and equivalents in form and function, as will occur to those ordinarily skilled in the pertinent arts having the benefit of this disclosure.

While the present invention has been described for what are presently considered the preferred embodiments, the invention is not so limited. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the detailed description provided above.

CLAIMS

We claim:

1. A method for enhancing cell growth of a cell in culture comprising the addition of a supplement to said cell culture medium, wherein said supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein said recombinant albumin is
   i) produced in a plant,
   ii) has less than about 1 EU of endotoxin / mg of albumin; and
   iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000, to about 1 to 0.5.

2. A method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to said serum free media, wherein said supplement comprises; a mixture of recombinant albumin and a transferrin related protein;wherein said recombinant albumin is
   i) produced in a plant.
   ii) has less than about 1 EU of endotoxin / mg of albumin; and
   iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000, to about 1 to 0.5.

3. A method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to said culture wherein said supplement comprises a mixture of recombinant albumin and a transferrin related protein:
   wherein said recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000 to about 1 to 0.5.

4. A method for improving the viability of cells in culture in a bioreactor comprising the addition of a supplement to said culture in said bioreactor wherein said supplement comprises;
   a mixture of recombinant albumin and a transferrin related protein;

wherein said recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000 to about 1 to 0.5.

5. A method for improving the viability of cells grown in serum free media comprising the addition of a supplement to said serum free media wherein said supplement comprises a mixture of recombinant albumin and a transferrin related protein;
wherein said recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000 to about 1 to 0.5.
wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5,000 to about 1 to 0.5.

6. The method claims 2 or 3, wherein the viability of the cell in culture is increased.

7. The method of any of claims 1 to 5, wherein said supplement further comprises insulin.

8. The method of any of claims 1 to 5, wherein said supplement further comprises, selenium and ethanolamine.

9. The method of any of claims 1-5, wherein said cells are tissue culture cells.

10. The method of any of claims 1-5, wherein said cells are CHO cells.

11. The method of any of claims 1-5, wherein said cells are hybridoma cells.

12. The method of claim 3, wherein said protein is an antibody.

13. The method of any of claims 1-5, wherein said recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein.

14. The method of claim 13, wherein said heat shock protein is a rice heat shock protein.

15. The method of claim 13, wherein said heat shock protein is selected from said group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein.

16. The method of claim 13, wherein said heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / Osl_37938, and AAB63469.

17. The method of claim 13, wherein said transferrin related protein is lactoferrin.

18. The method of claim 13, wherein said transferrin related protein is transferrin.

19. The method of claim 18, wherein said ratio of said transferrin to said recombinant albumin is about 1 to 50, to about 1 to 5000.

20. The method of claim 17, wherein said lactoferrin is recombinant lactoferrin.

21. The method of claim 20, wherein said ratio of said lactoferrin to said recombinant albumin is about 1 to 3, to about 1 to 0.33.

22. The method of any of claims 4 to 6 wherein said improvement in cell viability is greater than 30 % compared to the summed effect of said recombinant albumin and said transferrin related protein when added alone when measured under the same culture conditions.

23. A supplement comprising; a mixture of recombinant albumin and a transferrin related protein; wherein said recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein said supplement comprises a ratio (wt/wt) of said transferrin related protein to said recombinant albumin of about 1 to 5000 to about 1 to 0.5.

24. The supplement of claim 23, wherein said recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein.

25. The supplement of claim 24, wherein said heat shock protein is a rice heat shock protein.

26. The supplement of claim 24, wherein said heat shock protein is selected from said group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein.

27. The supplement of claim 24, wherein said heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / Osl_37938, and AAB63469.

28. The supplement of claim 24, wherein said supplement comprises at least about 0.04 % wt / wt HSP70.

29. The supplement of claim 24, wherein said transferrin related protein is lactoferrin.

30. The supplement of claim 24, wherein said transferrin related protein is transferrin.

31. The supplement of claim 30, wherein said ratio of said transferrin to said recombinant albumin is about 1 to 50, to about 1 to 5000.

32. The supplement of claim 31, wherein said ratio of said lactoferrin to said recombinant albumin is about 1 to 3, to about 1 to 0.33.

ABSTRACT OF THE DISCLOSURE

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

Figure 1A/20

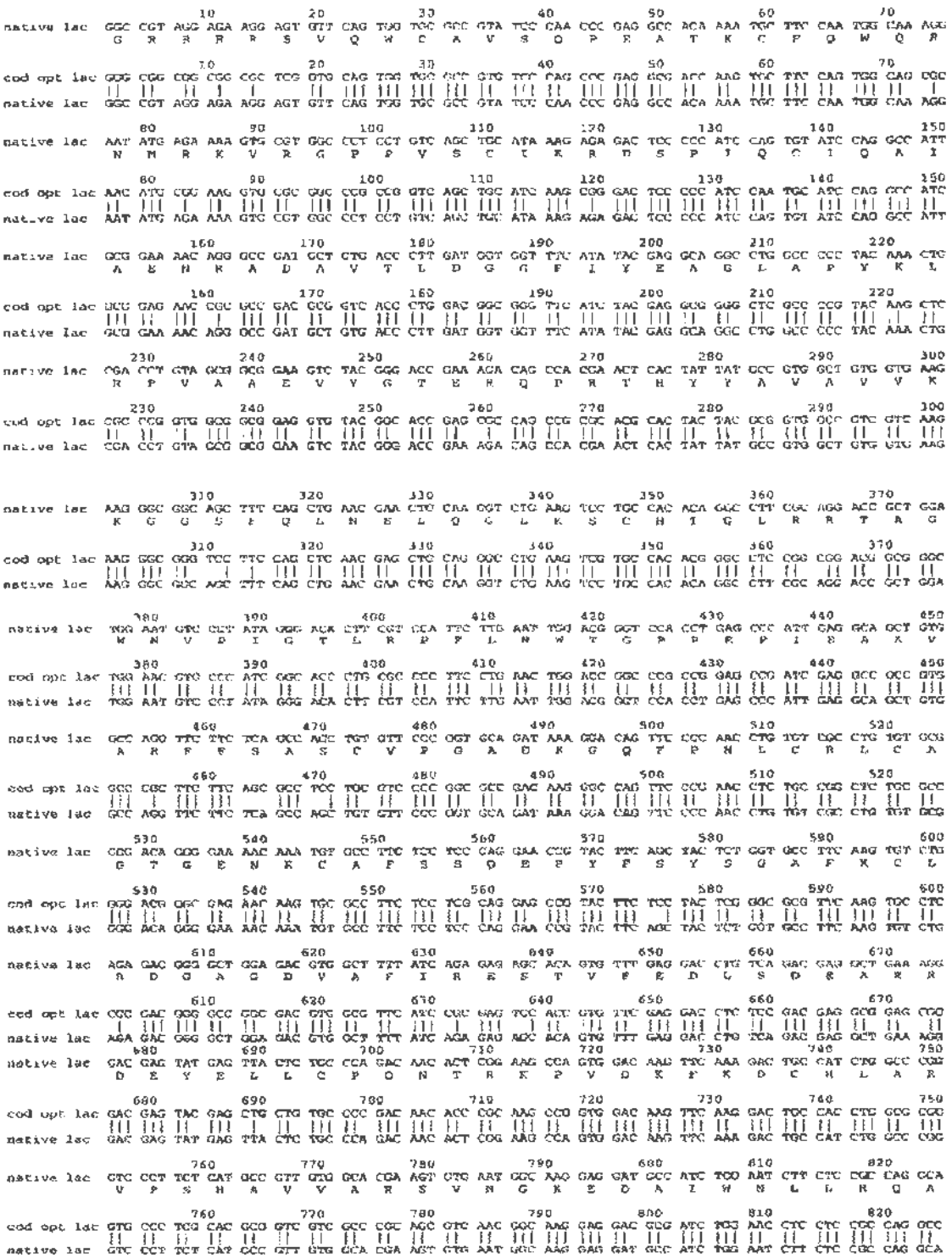


Figure 1B/20

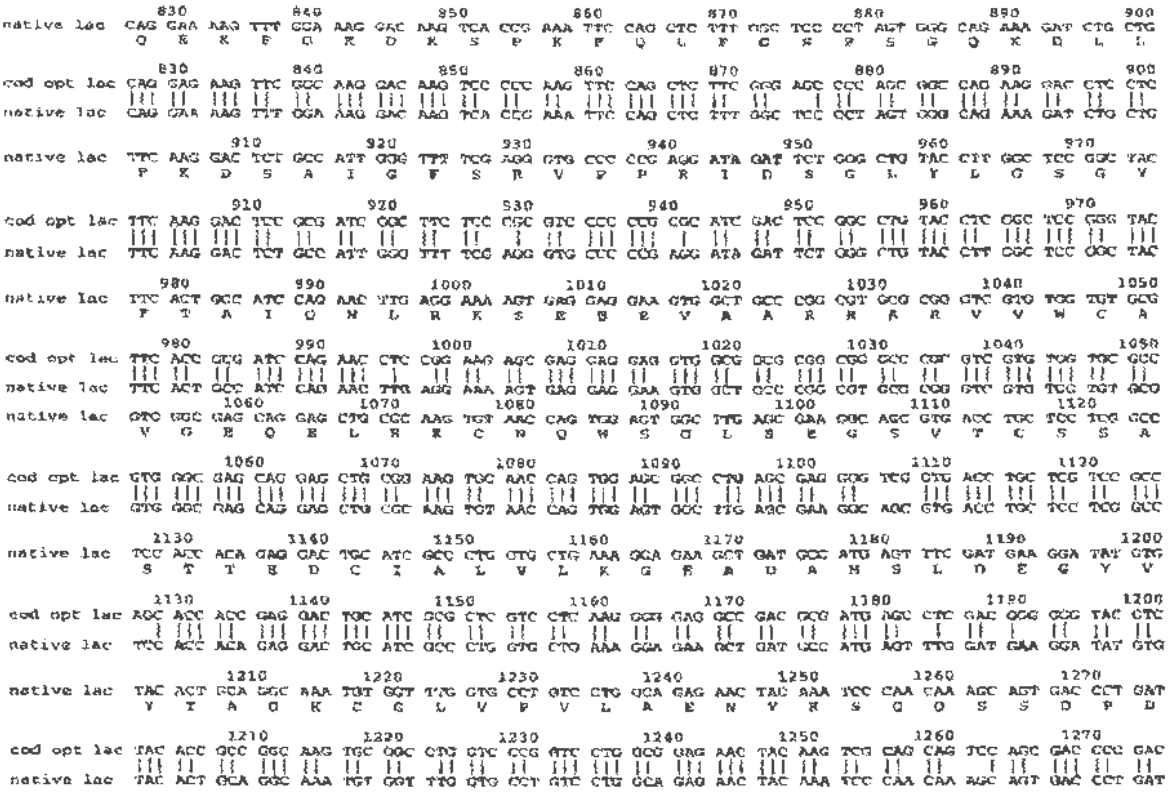


Figure 1C/20

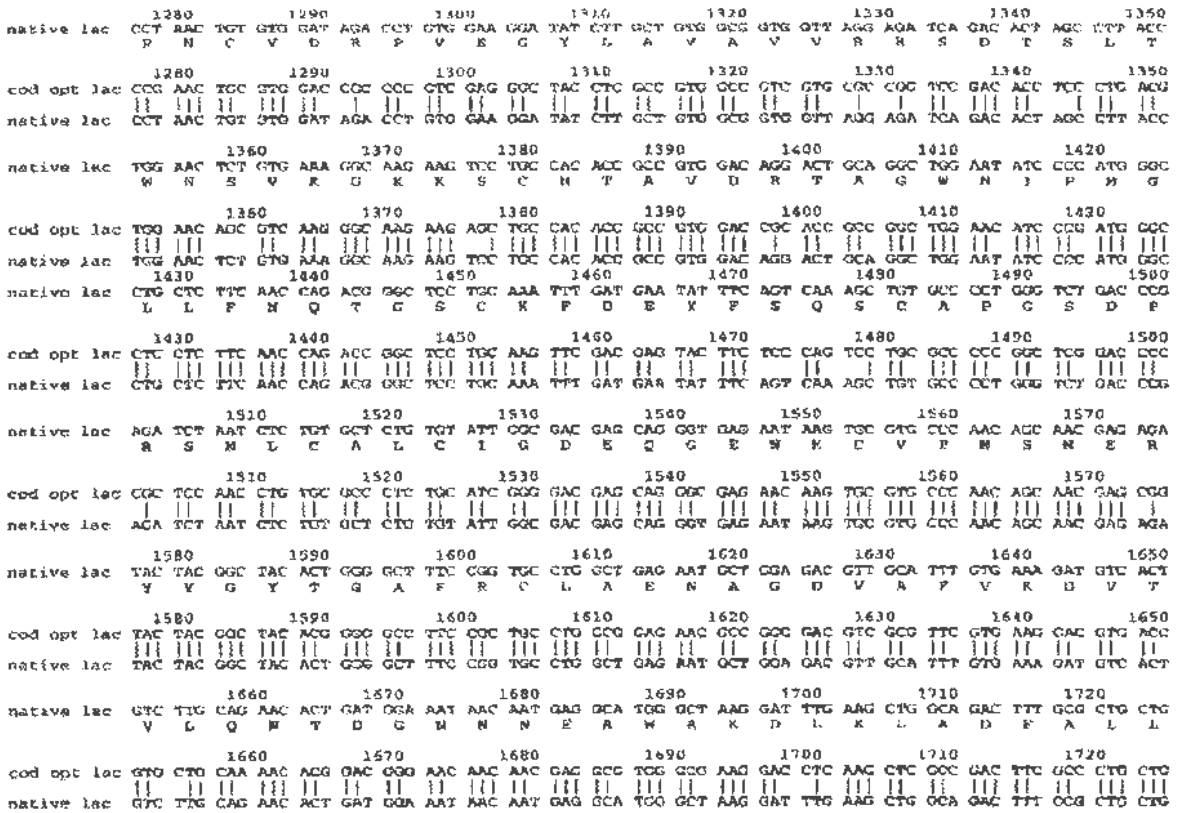


Figure 1D/20

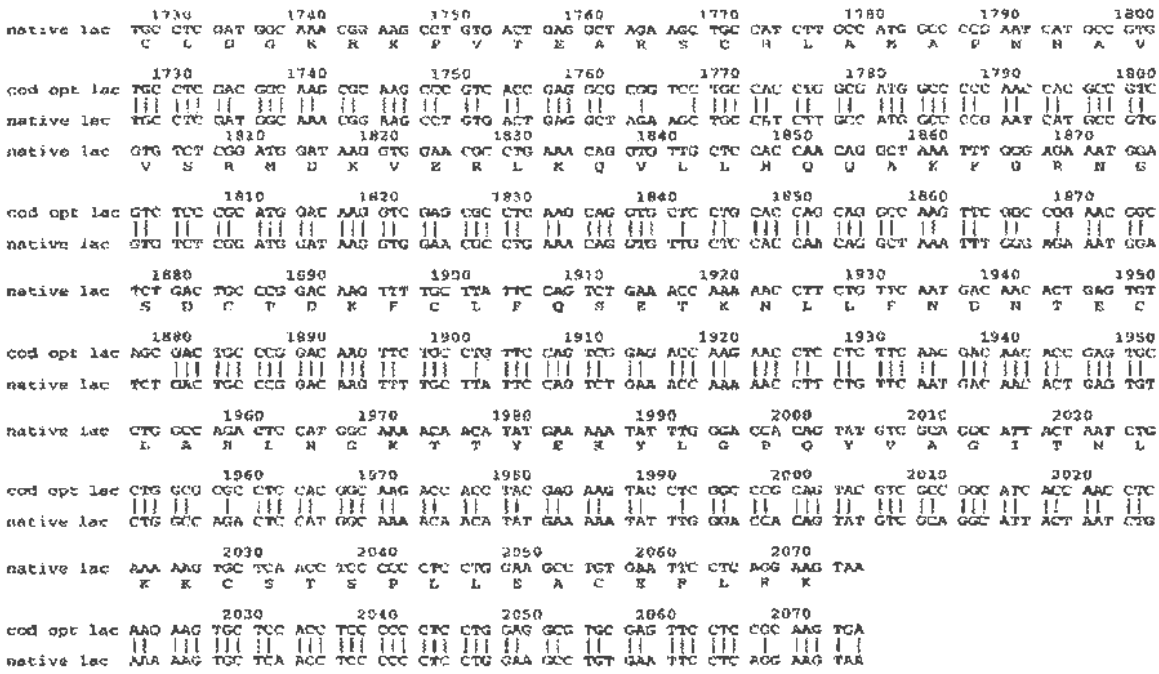

native lac
cod opt lac
2079 = Total number of nucleotides
467 = Number of nucleotides changed
693 = Total Number of codons
413 = Number of codons changed

Figure 2/20

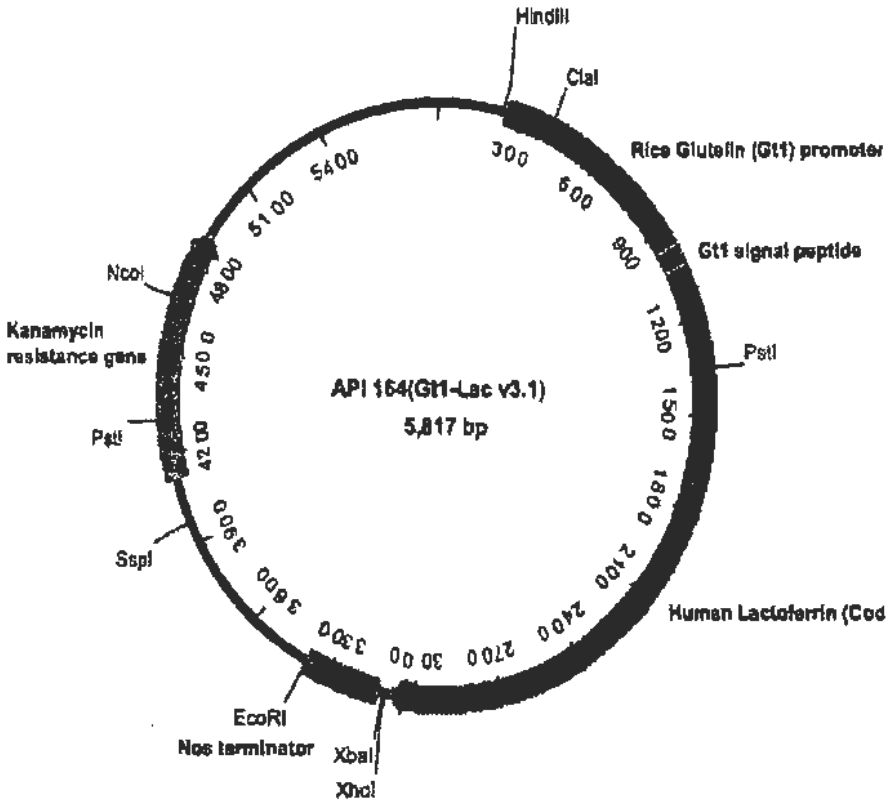

HindIII
ClaI
Rice Glutelin (Gt1) promoter
Gt1 signal peptide
PstI
Human Lactoferrin (Cod
XhoI
XbaI
EcoRI
Nos terminator
SspI
PstI
NcoI
Kanamycin
resistance gene
API 164(Gt1-Lac v3.1)
5,817 bp
300
600
900
1200
1500
1800
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4800
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Figure 3/20

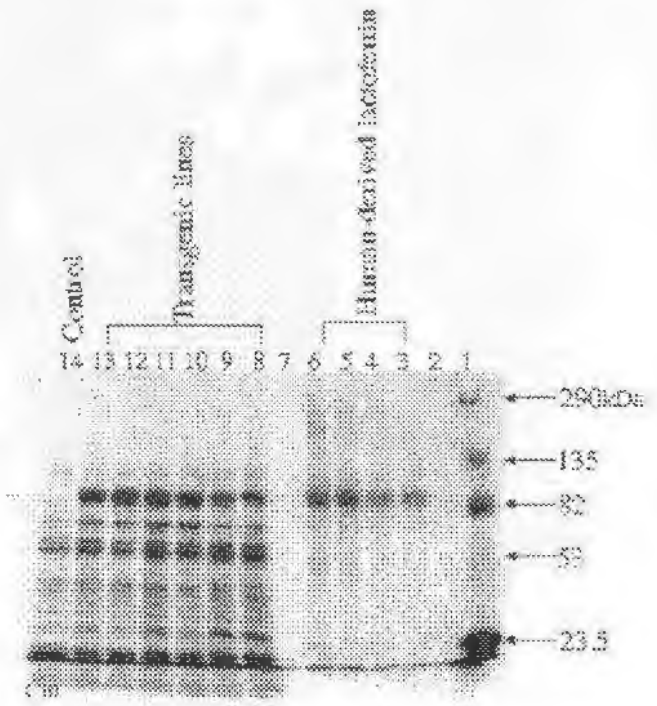

Control
Transgenic Lines
Bluetongue-derived Isolates
14 13 12 11 10 9 8 7 6 5 4 3 2 1
290kDa
135
82
53
23.5

Figure 4/20

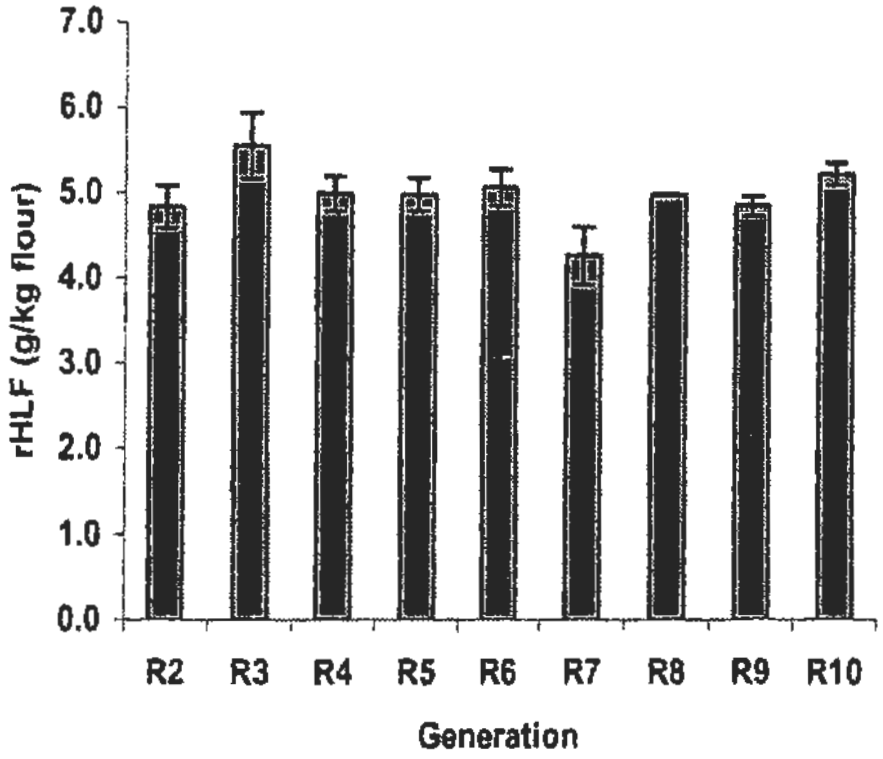

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
rHLF (g/Kg flour)
R2
R3
R4
R5
R6
R7
R8
R9
R10
Generation

Figure 5A/20

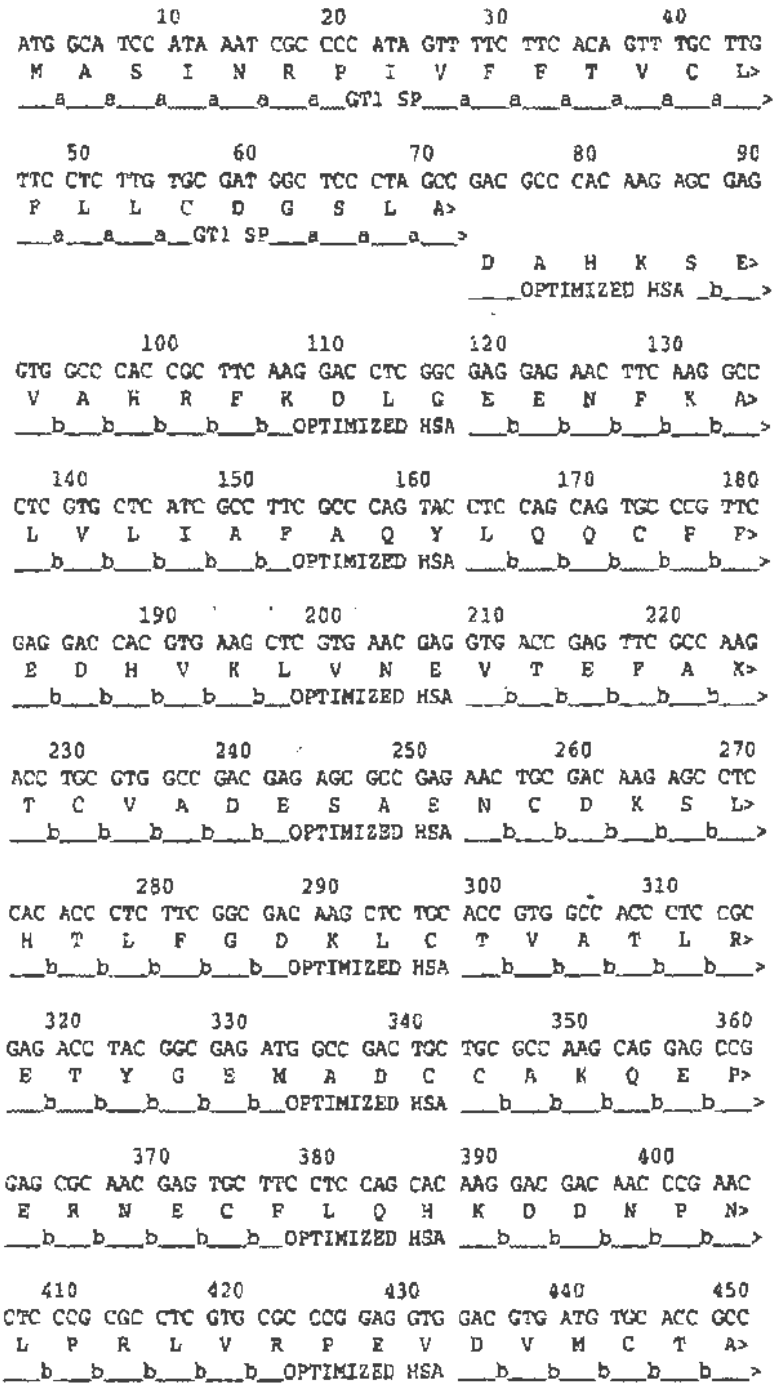

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F L L C D G S L A>
_a_a_a_GT1 SP_a_a_a_>
D A H K S E>
_OPTIMIZED HSA _b_>
100 110 120 130
GTG GCC CAC CGC TTC AAG GAC CTC GGC GAG GAG AAC TTC AAG GCC
V A H R F K D L G E E N F K A>
_b_b_b_b_b_OPTIMIZED HSA _b_b_b_b_b_>
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L V L I A F A Q Y L Q Q C P F>
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E D H V K L V N E V T E F A K>
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ACC TGC GTG GCC GAC GAG AGC GCC GAG AAC TGC GAC AAG AGC CTC
T C V A D E S A E N C D K S L>
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370 380 390 400
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E R N E C F L Q H K D D N P N>
_b_b_b_b_b_OPTIMIZED HSA _b_b_b_b_b_>
410 420 430 440 450
CTC CCG CGC CTC GTG CGC CCG GAG GTG GAC GTG ATG TGC ACC GCC
L P R L V R P E V D V M C T A>
_b_b_b_b_b_OPTIMIZED HSA _b_b_b_b_b_>

Figure 5 B/20

```
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 F   H   D   N   E   E   T   F   L   K   K   Y   L   Y   E>
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 I   A   R   R   H   P   Y   P   Y   A   P   E   L   L   F>
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TTC GCC AAG CGC TAC AAG GCC GCC TTC ACC GAG TGC TGC CAG GCC
 F   A   K   R   Y   K   A   A   F   T   E   C   C   Q   A>
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   590          600          610          620          630
GCC GAC AAG GCC GCC TGC CTC CTC CCG AAG CTC GAC GAG CTC CGC
 A   D   K   A   A   C   L   L   P   K   L   D   E   L   R>
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          640          650          660          670
GAC GAG GGC AAG GCC TCC AGC GCC AAG CAG CGC CTC AAG TGC GCC
 D   E   G   K   A   S   S   A   K   Q   R   L   K   C   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   680          690          700          710          720
AGC CTC CAG AAG TTC GGC GAG CGC GCC TTC AAG GCC TGG GCC GTG
 S   L   Q   K   F   G   E   R   A   F   K   A   W   A   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          730          740          750          760
GCC CGC CTC AGC CAG CGC TTC CCG AAG GCC GAG TTC GCC GAG GTG
 A   R   L   S   Q   R   F   P   K   A   E   F   A   E   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   770          780          790          800          810
TCC AAG CTC GTG ACC GAC CTC ACC AAG GTG CAC ACC GAG TGC TGC
 S   K   L   V   T   D   L   T   K   V   H   T   E   C   C>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          820          830          840          850
CAC GGC GAC CTC CTG GAG TGC GCC GAC GAC CGC GCC GAC CTC GCC
 H   G   D   L   L   E   C   A   D   D   R   A   D   L   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   860          870          880          890          900
AAG TAC ATC TGC GAG AAC CAG GAC AGC ATC TCC AGC AAG CTC AAG
 K   Y   I   C   E   N   Q   D   S   I   S   S   K   L   K>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          910          920          930          940
GAG TGC TGC GAG AAG CCG CTC CTG GAG AAG TCC CAC TGC ATC GCC
 E   C   C   E   K   P   L   L   E   K   S   H   C   I   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
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Figure 5C/20

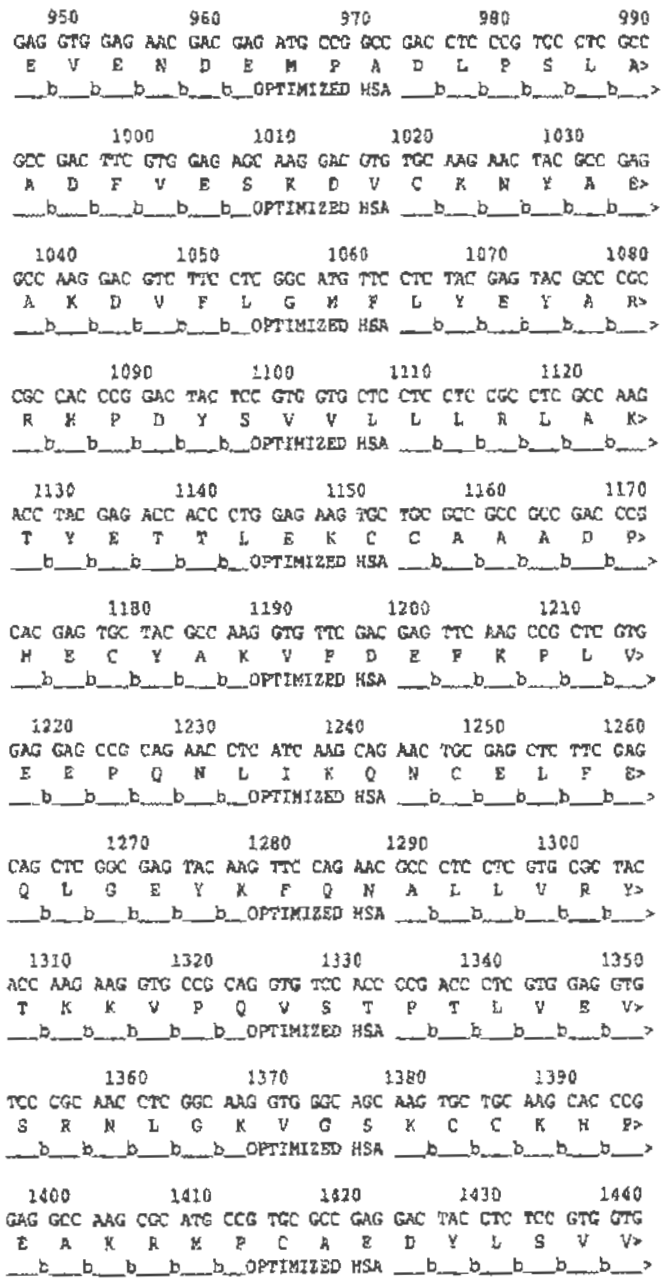

950 960 970 980 990
GAG GTG GAG AAC GAC GAG ATG CCG GCC GAC CTC CCG TCC CTC GCC
E V E N D E M P A D L P S L A>
OPTIMIZED HSA
1000 1010 1020 1030
GCC GAC TTC GTG GAG AGC AAG GAC GTG TGC AAG AAC TAC GCC GAG
A D F V E S K D V C K N Y A E>
OPTIMIZED HSA
1040 1050 1060 1070 1080
GCC AAG GAC GTC TTC CTC GGC ATG TTC CTC TAC GAG TAC GCC CGC
A K D V F L G M F L Y E Y A R>
OPTIMIZED HSA
1090 1100 1110 1120
CGC CAC CCG GAC TAC TCC GTG GTG CTC CTC CTC CGC CTC GCC AAG
R H P D Y S V V L L L R L A K>
OPTIMIZED HSA
1130 1140 1150 1160 1170
ACC TAC GAG ACC ACC CTG GAG AAG TGC TGC GCC GCC GCC GAC CCG
T Y E T T L E K C C A A A D P>
OPTIMIZED HSA
1180 1190 1200 1210
CAC GAG TGC TAC GCC AAG GTG TTC GAC GAG TTC AAG CCG CTC GTG
H E C Y A K V F D E F K P L V>
OPTIMIZED HSA
1220 1230 1240 1250 1260
GAG GAG CCG CAG AAC CTC ATC AAG CAG AAC TGC GAG CTC TTC GAG
E E P Q N L I K Q N C E L F E>
OPTIMIZED HSA
1270 1280 1290 1300
CAG CTC GGC GAG TAC AAG TTC CAG AAC GCC CTC CTC GTG CGC TAC
Q L G E Y K F Q N A L L V R Y>
OPTIMIZED HSA
1310 1320 1330 1340 1350
ACC AAG AAG GTG CCG CAG GTG TCC ACC CCG ACC CTC GTG GAG GTG
T K K V P Q V S T P T L V E V>
OPTIMIZED HSA
1360 1370 1380 1390
TCC CGC AAC CTC GGC AAG GTG GGC AGC AAG TGC TGC AAG CAC CCG
S R N L G K V G S K C C K H P>
OPTIMIZED HSA
1400 1410 1420 1430 1440
GAG GCC AAG CGC ATG CCG TGC GCC GAG GAC TAC CTC TCC GTG GTG
E A K R M P C A E D Y L S V V>
OPTIMIZED HSA

Figure 5D/20

```
         1450         1460         1470         1480
CTC AAC CAG CTC TGC GTG CTC CAC GAG AAG ACC CCG GTG AGC GAC
 L   N   Q   L   C   V   L   H   E.  K   T   P   V   S   D>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1490         1500         1510         1520         1530
CGC GTG ACC AAG TGC TGC ACC GAG AGC CTC GTG AAC CGC CGC CCG
 R   V   T   K   C   C   T   E   S   L   V   N   R   R   P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1540         1550         1560         1570
TGC TTC TCC GCC CTG GAG GTC GAC GAG ACC TAC GTC CCG AAG GAG
 C   F   S   A   L   E   V   D   E   T   Y   V   P   K   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1580         1590         1600         1610         1620
TTC AAC GCC GAG ACC TTC ACC TTC CAC GCC GAC ATC TGC ACC CTC
 F   N   A   E   T   F   T   F   H   A   D   I   C   T   L>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1630         1640         1650         1660
TCC GAG AAG GAG CGC CAG ATC AAG AAG CAG ACC GCC CTC GTC GAG
 S   E   K   E   R   Q   I   K   K   Q   T   A   L   V   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1670         1680         1690         1700         1710
CTC GTG AAG CAC AAG CCG AAG GCC ACC AAG GAG CAG CTC AAG GCC
 L   V   K   H   K   P   K   A   T   K   E   Q   L   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1720         1730         1740         1750
GTG ATG GAC GAC TTC GCC GCC TTC GTG GAG AAG TGC TGC AAG GCC
 V   M   D   D   F   A   A   F   V   E   K   C   C   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1760         1770         1780         1790         1800
GAC GAC AAG GAG ACC TGC TTC GCC GAG GAG GGC AAG AAG CTC GTG
 D   D   K   E   T   C   F   A   E   E   G   K   K   L   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1810         1820         1830
GCC GCC AGC CAG GCC GCC CTC GGC CTC TGA
 A   A   S   Q   A   A   L   G   L   *>
___b___b____OPTIMIZED HSA _b___b___b___>
```

Figure 6/20

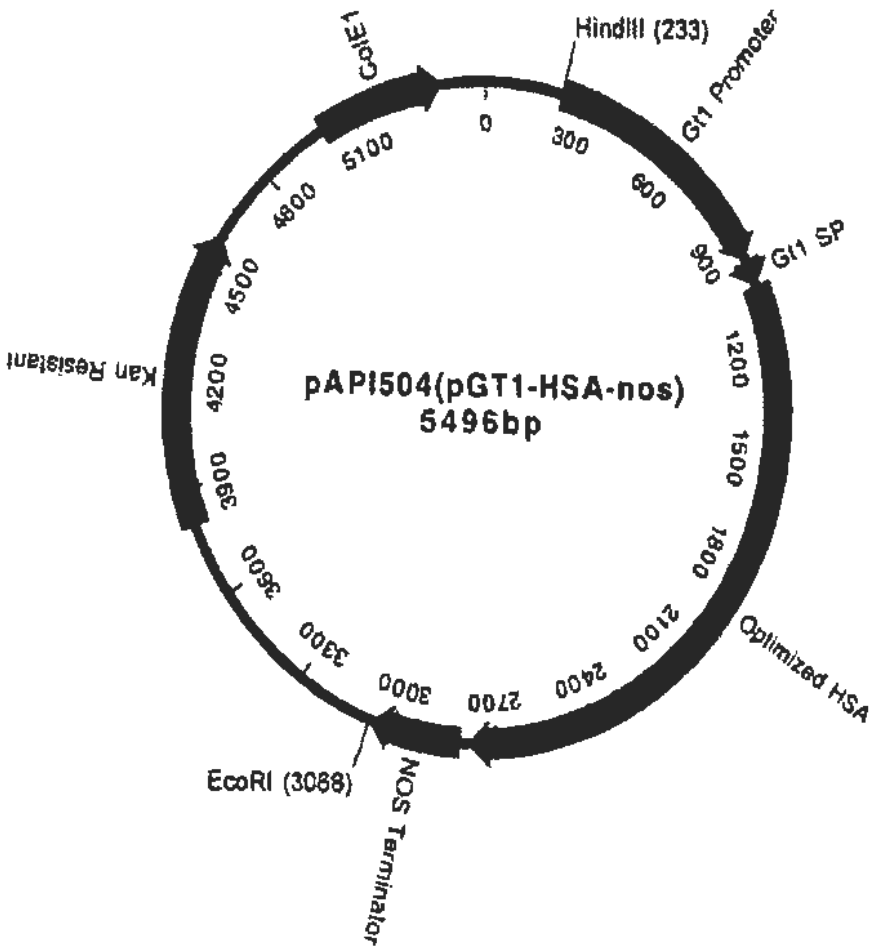

pAPI504(pGT1-HSA-nos)
5496bp
ColE1
HindIII (233)
Gt1 Promoter
Gt1 SP
Optimized HSA
NOS Terminator
EcoRI (3088)
Kan Resistant
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
3600
3900
4200
4500
4800
5100

Figure 7/20

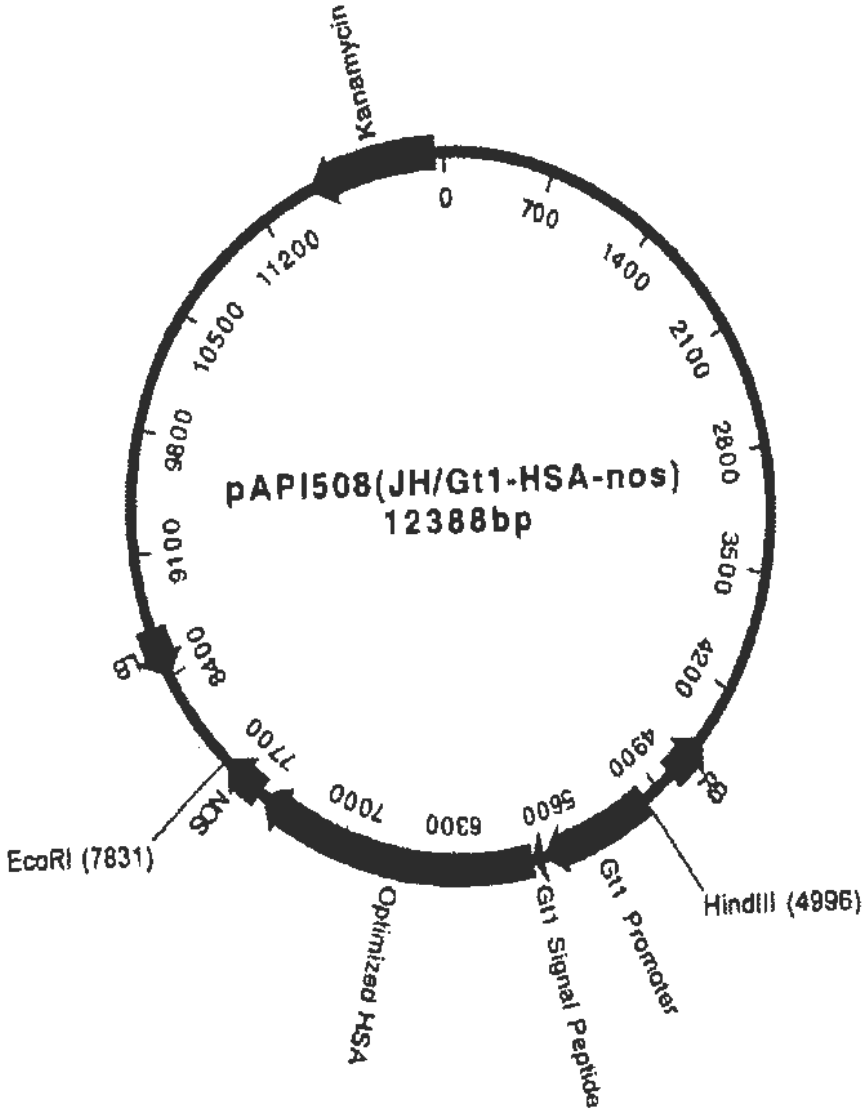
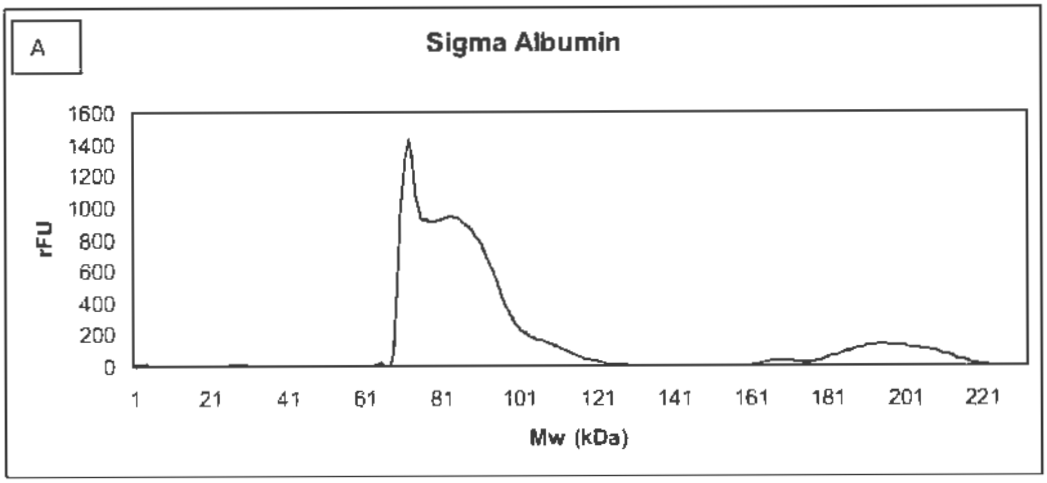
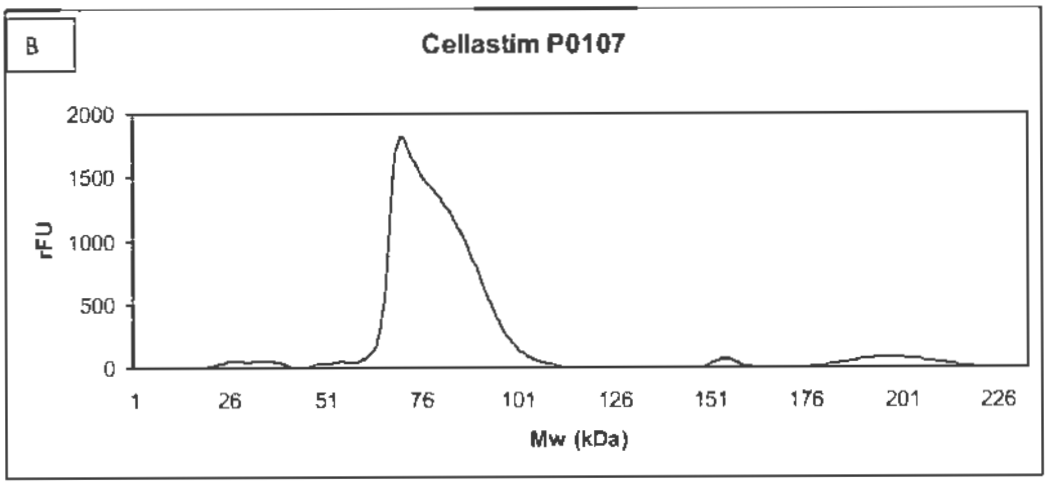

pAPI508(JH/Gt1-HSA-nos)
12388bp
Kanamycin
0
700
1400
2100
2800
3500
4200
4900
5600
6300
7000
7700
8400
9100
9800
10500
11200
pB
Gt1 Promoter
Gt1 Signal Peptide
Optimized HSA
NOS
pA
HindIII (4996)
EcoRI (7831)

Figure 8/20


A
Sigma Albumin
rFU
1600
1400
1200
1000
800
600
400
200
0
1
21
41
61
81
101
121
141
161
181
201
221
Mw (kDa)


B
Cellastim P0107
rFU
2000
1500
1000
500
0
1
26
51
76
101
126
151
176
201
226
Mw (kDa)

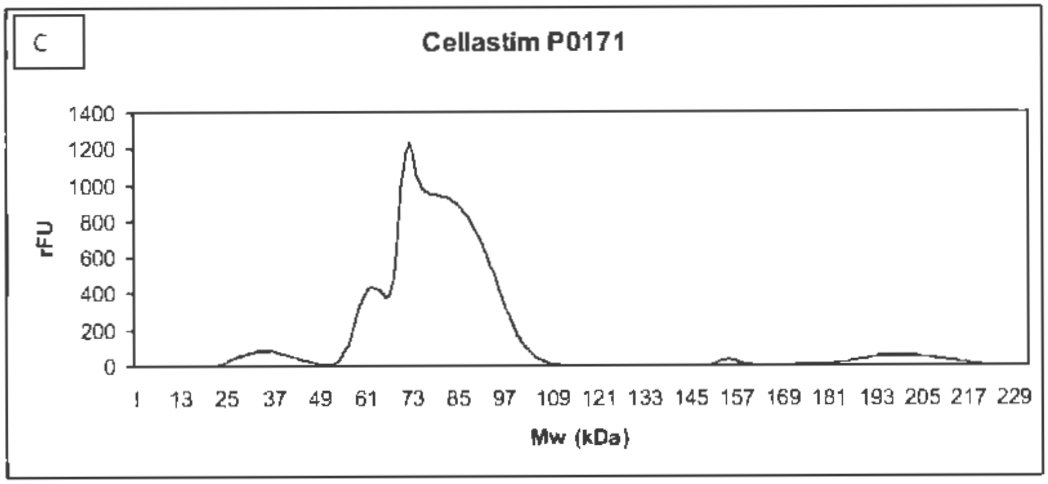
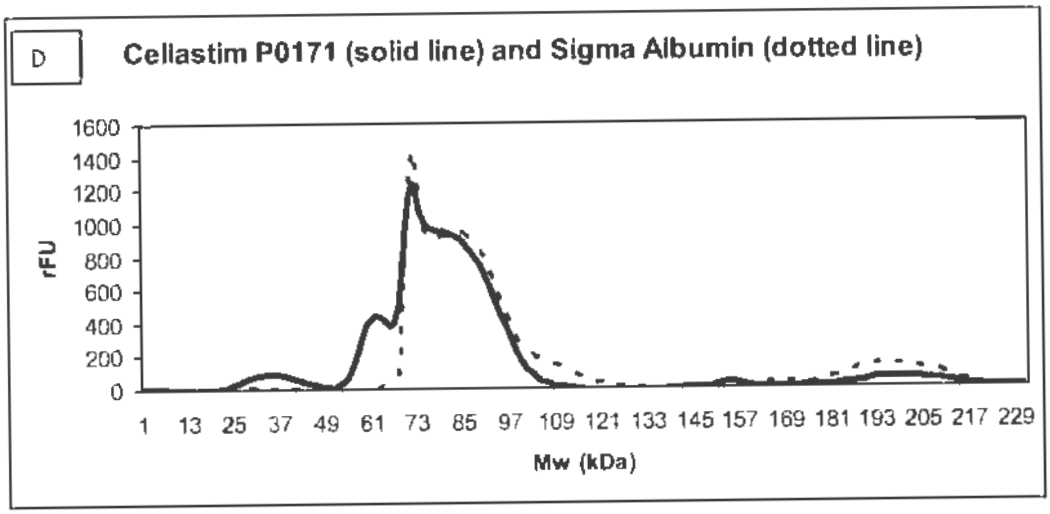

C
Cellastim P0171
rFU
1400
1200
1000
800
600
400
200
0
1
13
25
37
49
61
73
85
97
109
121
133
145
157
169
181
193
205
217
229
Mw (kDa)

Figure 8(II)/20



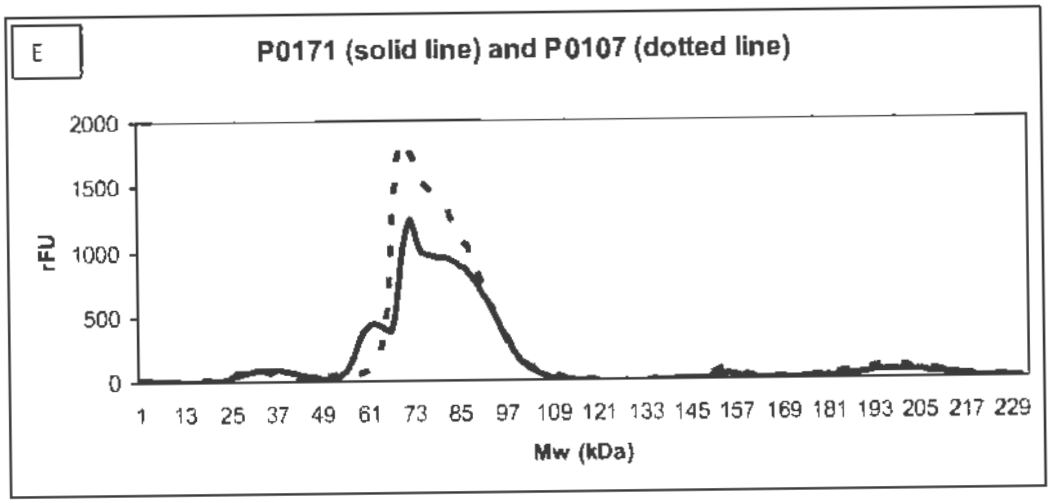
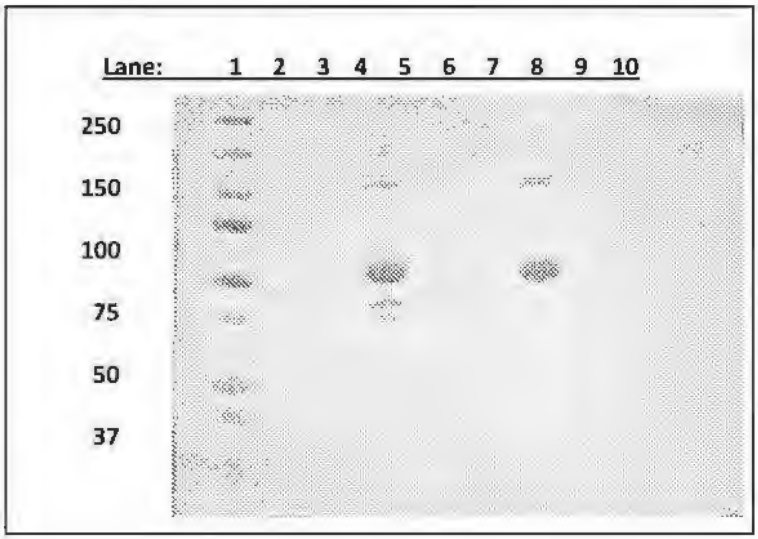


Figure 9/20


Lane: 1 2 3 4 5 6 7 8 9 10
250
150
100
75
50
37

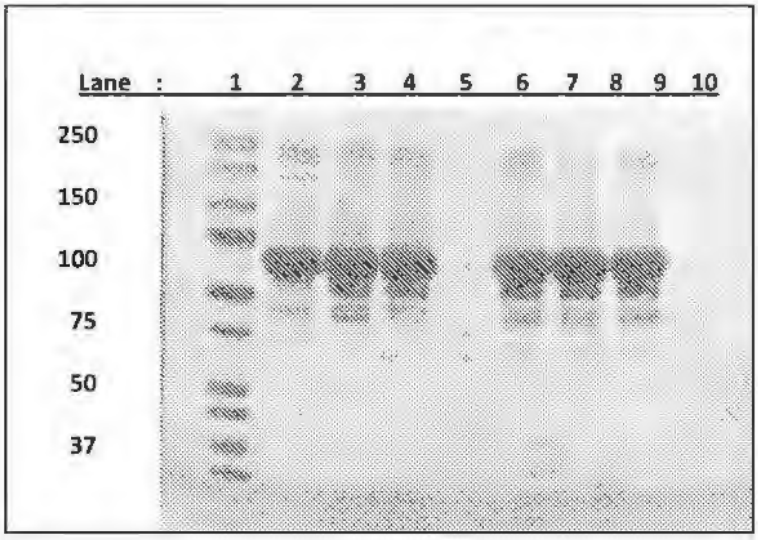
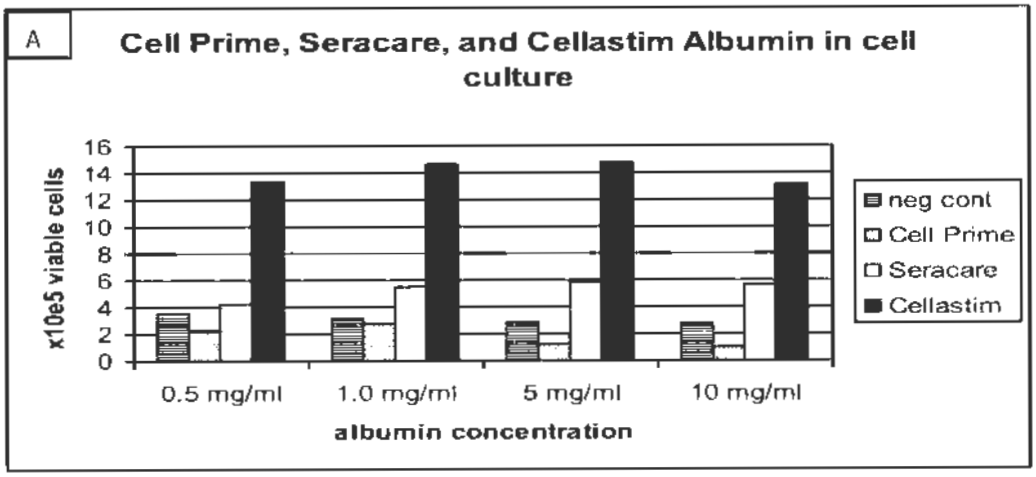
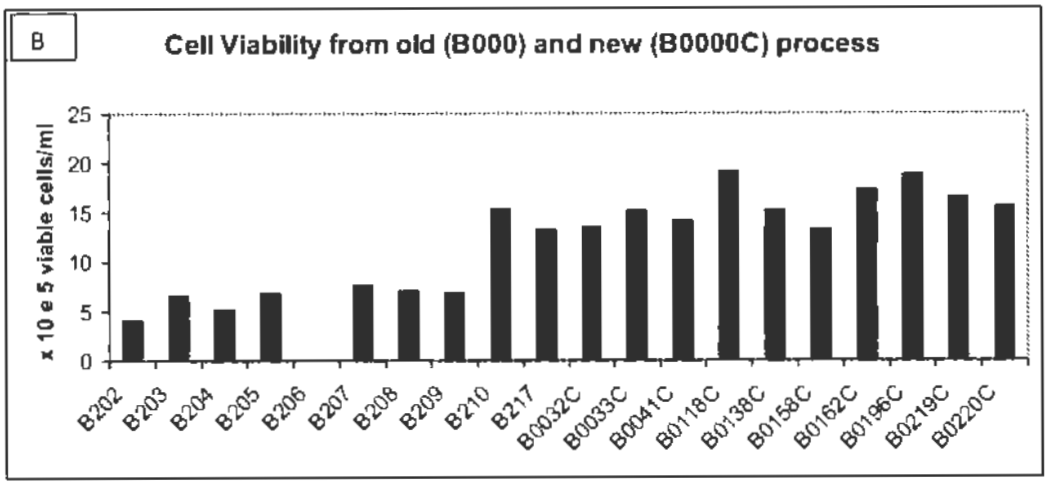

Lane : 1 2 3 4 5 6 7 8 9 10
250
150
100
75
50
37

Figure 10/20


A
Cell Prime, Seracare, and Cellastim Albumin in cell culture
x10e5 viable cells
16
14
12
10
8
6
4
2
0
0.5 mg/ml
1.0 mg/ml
5 mg/ml
10 mg/ml
albumin concentration
neg cont
Cell Prime
Seracare
Cellastim


B
Cell Viability from old (B000) and new (B0000C) process
x 10 e 5 viable cells/ml
25
20
15
10
5
0
B202
B203
B204
B205
B206
B207
B208
B209
B210
B217
B0032C
B0033C
B0041C
B0118C
B0138C
B0158C
B0162C
B0196C
B0219C
B0220C

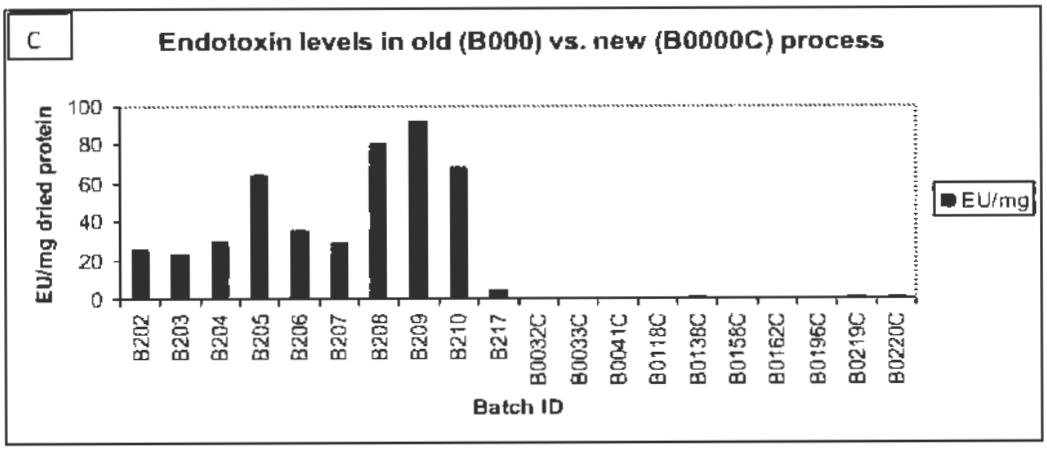

C
Endotoxin levels in old (B000) vs. new (B0000C) process
EU/mg dried protein
100
80
60
40
20
0
B202
B203
B204
B205
B206
B207
B208
B209
B210
B217
B0032C
B0033C
B0041C
B0118C
B0138C
B0158C
B0162C
B0196C
B0219C
B0220C
Batch ID
EU/mg

Figure 11/20

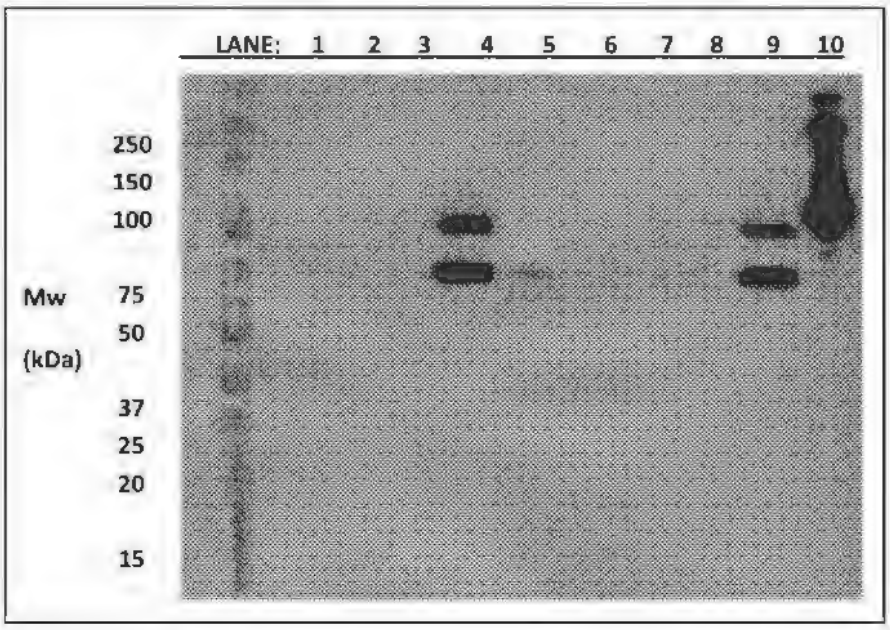


Figure 12/20

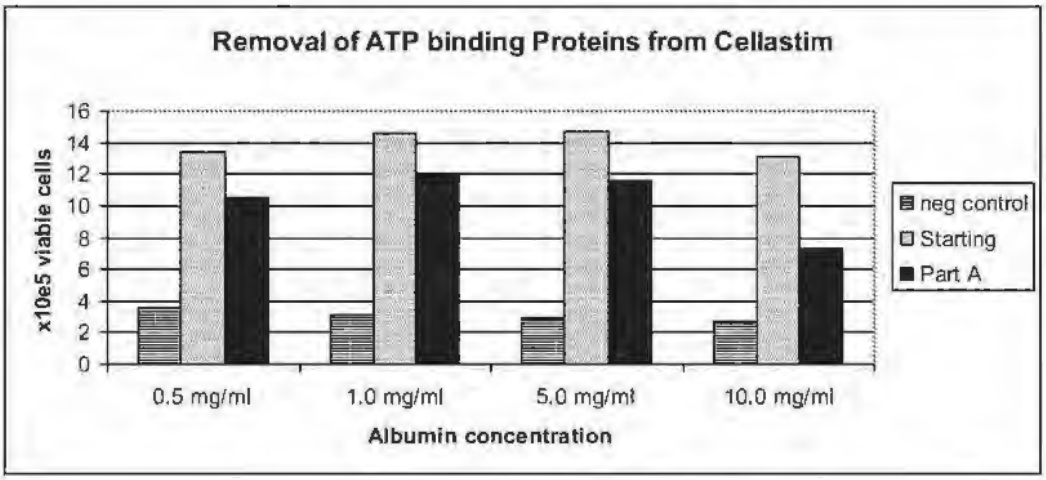
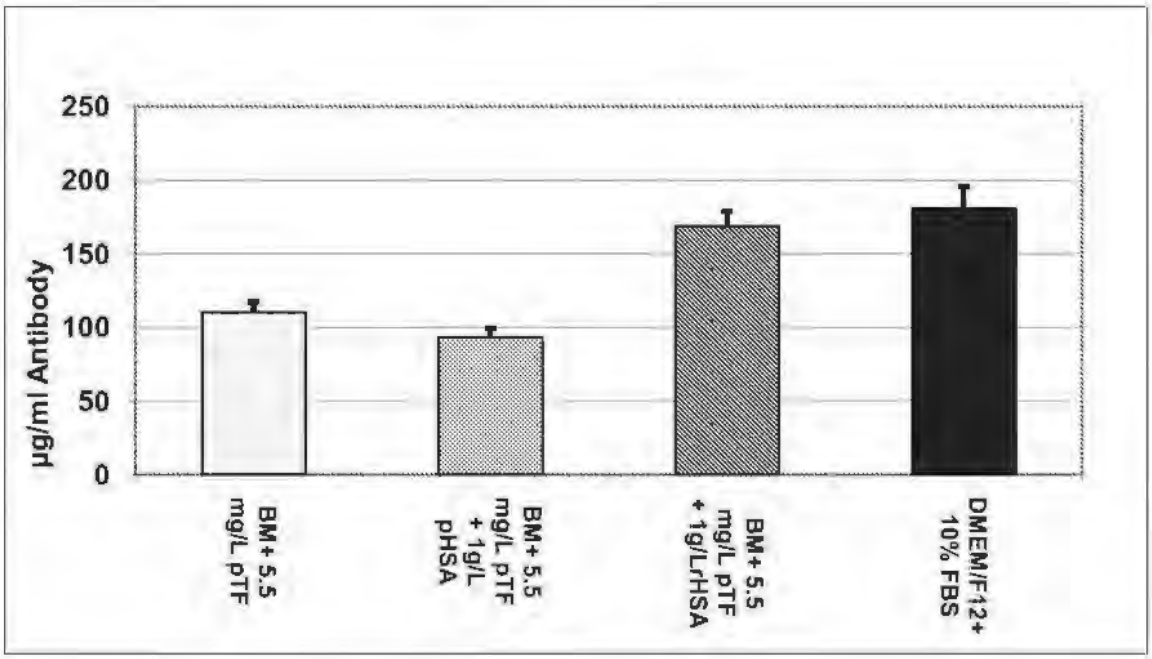

Removal of ATP binding Proteins from Cellastim
x10e5 viable cells
16
14
12
10
8
6
4
2
0
0.5 mg/ml
1.0 mg/ml
5.0 mg/ml
10.0 mg/ml
Albumin concentration
neg control
Starting
Part A

Figure 13/20


250
200
150
100
50
0
µg/ml Antibody
BM+ 5.5 mg/L pTF
BM+ 5.5 mg/L pTF + 1g/L pHSA
BM+ 5.5 mg/L pTF + 1g/LrHSA
DMEM/F12+ 10% FBS

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

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☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

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Postal Code	80525	Country i	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Ning		Huang	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

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City	Fort Collins	**State/Province**	CO
Postal Code	80525	**Country**	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	72659		
Email Address	admin@globalpatentgroup.com	Add Email	Remove Email

Application Information:

Title of the Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS		
Attorney Docket Number	VBS0002-201C1-US	**Small Entity Status Claimed**	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	20	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

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Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	• Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	72659		

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This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	12708462	2010-02-18
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
12708462	Claims benefit of provisional	61154204	2009-02-20

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[1] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

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Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017 OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
● Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name: Ventria BioScience Inc.

Mailing Address Information For Applicant:

Address 1	2120 Milestone Drive		
Address 2	Suite 102		
City	Fort Collins	**State/Province**	CO
Country	US	Postal Code	80525
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

PTO/AIA/14 (11-15)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		State/Province	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Dennis A. Bennett/			Date (YYYY-MM-DD)	2016-06-21
First Name	Dennis	Last Name	Bennett	Registration Number	34547

Additional Signature may be generated within this form by selecting the Add button. Add

PTO/AIA/14 (11-15)
Approved for use through 04/30/2017 OMB 0651-0032
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	VBS0002-201C1-US
	Application Number	
Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	26130631
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	72659
Filer:	Dennis A. Bennett/Vicki Truman
Filer Authorized By:	Dennis A. Bennett
Attorney Docket Number:	VBS0002-201C1-US
Receipt Date:	21-JUN-2016
Filing Date:	
Time Stamp:	16:17:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		20160621_Application.pdf	804411	yes	138

	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	133	
	Claims		134	137	
	Abstract		138	138	
Warnings:					
Information:					
2	Drawings-only black and white line drawings	20160621_Figures.pdf	4197065	no	20
Warnings:					
Information:					
3	Sequence Listing (Text File)	20160621_Sequence_Listing.txt	5911	no	0
Warnings:					
Information:					
4	Application Data Sheet	20160621_ADS.pdf	1823170	no	9
Warnings:					
Information:					
		Total Files Size (in bytes):	6830557		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

SCORE Placeholder Sheet for IFW Content

Application Number: 15188478 Document Date: 06/21/2016

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The IFW images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions below.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface
- Other USPTO employees can bookmark the current SCORE URL (http://Score.uspto.gov/ScoreAccessWeb/).
- External customers may access SCORE content via the Public and Private PAIR interfaces.

Form Revision Date: September 30, 2013

```
============================================================
Sequence Listing could not be accepted due to errors.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Douglas, Keisha (ASRC)
Timestamp: [year=2016; month=6; day=28; hr=15; min=45; sec=54; ms=535; ]
------------------------------------------------------------


************************************************************
Reviewer Comments:
1. For the following error codes and descriptions, an explanation follows
below.


Error Code        Error Description
E287            Invalid WIPO ST.2 date format; Use (YYYY-MM-DD) in <141>
E287            Invalid WIPO ST.2 date format; Use (YYYY-MM-DD)in <151>
E248            Order Sequence Error <151> -> <213>; Expected Mandatory
Tag: <210> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
W112            Upper case found in data; Found at position(0) SeqId(0)
W112            Upper case found in data; Found at position(1) SeqId(0)
W112            Upper case found in data; Found at position(2) SeqId(0)
W112            Upper case found in data; Found at position(3) SeqId(0)
W112            Upper case found in data; Found at position(4) SeqId(0)
W112            Upper case found in data; Found at position(5) SeqId(0)
W112            Upper case found in data; Found at position(6) SeqId(0)
W112            Upper case found in data; Found at position(7) SeqId(0)
W112            Upper case found in data; Found at position(8) SeqId(0)
W112            Upper case found in data; Found at position(9) SeqId(0)
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W112            Upper case found in data; Found at position(14) SeqId(0)
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W112            Upper case found in data; Found at position(17) SeqId(0)
W112            Upper case found in data; Found at position(18) SeqId(0)
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W112            Upper case found in data; Found at position(19) SeqId(0)
This error has occured more than 20 times, will not be displayed
E444            Invalid line termination: on line 29 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 31 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 33 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 35 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 37 and tag: <400>
line length validation was not performed
E444            Invalid line termination: on line 39 and tag: <400>
line length validation was not performed
E444            Invalid line termination: on line 41 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 43 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 45 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 47 and tag: <400> -
line length validation was not performed
W446            The current line: 49 contains 75-80 characters in tag
<400> and Sequence ID (0)
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
E444            Invalid line termination: on line 59 and tag: <400> -
line length validation was not performed
W446            The current line: 61 contains 75-80 characters in tag
<400> and Sequence ID (0)
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
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Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
E444            Invalid line termination: on line 71 and tag: <400> -
line length validation was not performed
W446            The current line: 73 contains 75-80 characters in tag
<400> and Sequence ID (0)
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
E444            Invalid line termination: on line 83 and tag: <400> -
line length validation was not performed
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E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
E444            Invalid line termination: on line 95 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 97 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 99 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 101 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 103 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 105 and tag: <400> -
line length validation was not performed
E444            Invalid line termination: on line 107 and tag: <400> -
line length validation was not performed This error has occured more
than 20 times, will not be displayed
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
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E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
W446            The current line: 149 contains 75-80 characters in tag
<400> and Sequence ID (0)
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> <213>; Expected Mandatory
Tag: <212> in Header
W402            Undefined organism found in <213> in SEQ ID (0)
E310            Invalid sequence type in <212> in SEQID: (0)
E248            Order Sequence Error <212> -> <211>; Expected Mandatory
Tag: <213> in Header
E202            Invalid input format; Value must be an integer in <211>
in SEQID (0)
E248            Order Sequence Error <211> -> ; Expected Mandatory Tag:
<212> in Header
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The submitted file is a PatentIn "Work in Progress" file which is not properly formatted. The "Work in Progress" file must be converted by PatentIn to the final, properly formatted ".txt" (ASCII text) file. Please follow the following steps to generate a properly formatted .txt file:

1) Please open the ".prj" file in PatentIn, and go to "Application Steps" on the PatentIn main menu.

2) Scroll down and click on "Generate Sequence Listing". Click on the "Start" tab; the sequences will be generated, and you should see "Successful completion" in the blank section above the "Start" tab.

3) Go to "Project" on the main PatentIn menu, and scroll down to "View/Print Sequence Listing". You will then be able to see the generated PatentIn text file: this will be in the required ASCII text file format, having a ".txt" file extension. That is the file to submit

to the United States Patent and Trademark Office.

All Sequence Listing file line terminators must be "ASCII Carriage Return plus ASCII Line Feed," i.e., a "return" and a "space". Non-ASCII characters such as "Tabs" are not permitted.

There are over 20 improper line terminators in this Sequence Listing file. The first 17 are listed above. Please change the line terminators in this file to the correct "ASCII Carriage Return plus ASCII Line Feed," i.e., replace all tabs and non-ASCII characters with "space" or "return."

Applicants may consider creating an entirely new Sequence Listing in PatentIn rather than attempting to correct the extensive errors in the existing file. The US Patent and Trademark Office has created software, PatentIn, to assist applicants in preparing Sequence Listings in the proper format. The software and instruction manual are available for download at no cost from the USPTO website:

http://www.uspto.gov/patents/resources/tools/checker/patentinrel.jsp

To prevent line terminator errors in subsequent sequence listings, DO NOT edit the .txt file using word processing programs such as Microsoft Word. Word processing programs can introduce non-ASCII formatting into the file that while not visible, can be carried over into the .txt file and result in line terminator errors. Only edit the .txt file in programs such as Notepad or Wordpad.

Note:

The errors listed above are exemplary and may not be a complete list of all errors in the sequence listing file. The Applicant is required to review the sequence listing in its entirety and correct all instances of similar errors.

To correct the sequence listing errors noted in this report - The recommended method for correction of errors is to access the sequence listing working file using the software program in which the listing was

originally prepared, e.g., the project file in PatentIn, make any necessary corrections within that program, then generate a new sequence listing file. Use of a word processing program to correct errors directly in the original sequence listing file is strongly discouraged, since such programs often introduce unintended changes to the sequence listing, rendering the listing unacceptable. When the working file or original program is not available for correction, then use of a common or plain text-only editor, such as NotePad, to edit the original sequence listing file may suffice.

A sequence listing text file submitted via EFS-Web that complies with the requirements of 37 CFR 1.824(a) (2) (6) and (b) (i.e., is a compliant sequence listing ASCII text file), serves as both the paper copy required by 37 CFR 1.821(c) and the CRF required by 37 CFR 1.821(e).

****

Validated By CRFValidator v 1.0.5

Application No: 15188478 Version No: 1.0

Input Set:

Output Set:

Started: 2016-06-21 16:17:24.254
Finished: 2016-06-21 16:17:28.939
Elapsed: 0 hr(s) 0 min(s) 4 sec(s) 685 ms
Total Warnings: 2869
Total Errors: 78
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code		Error Description
E	287	Invalid WIPO ST.2 date format; Use (YYYY-MM-DD) in <141>
E	287	Invalid WIPO ST.2 date format; Use (YYYY-MM-DD)in <151>
E	248	Order Sequence Error <151> -> <213>; Expected Mandatory Tag: <210> in Header
W	402	Undefined organism found in <213> in SEQ ID (0)
W	112	Upper case found in data; Found at position(0) SeqId(0)
W	112	Upper case found in data; Found at position(1) SeqId(0)
W	112	Upper case found in data; Found at position(2) SeqId(0)
W	112	Upper case found in data; Found at position(3) SeqId(0)
W	112	Upper case found in data; Found at position(4) SeqId(0)
W	112	Upper case found in data; Found at position(5) SeqId(0)
W	112	Upper case found in data; Found at position(6) SeqId(0)
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W	112	Upper case found in data; Found at position(14) SeqId(0)

Input Set:

Output Set:

Started: 2016-06-21 16:17:24.254
Finished: 2016-06-21 16:17:28.939
Elapsed: 0 hr(s) 0 min(s) 4 sec(s) 685 ms
Total Warnings: 2869
Total Errors: 78
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code	Error Description
W 112	Upper case found in data; Found at position(15) SeqId(0)
W 112	Upper case found in data; Found at position(16) SeqId(0)
W 112	Upper case found in data; Found at position(17) SeqId(0)
W 112	Upper case found in data; Found at position(18) SeqId(0)
W 112	Upper case found in data; Found at position(19) SeqId(0) This error has occured more than 20 times, will not be displayed
E 444	Invalid line termination: on line 29 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 31 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 33 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 35 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 37 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 39 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 41 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 43 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 45 and tag: <400> - line length validation was not performed
E 444	Invalid line termination: on line 47 and tag: <400> - line length validation was not performed
W 446	The current line: 49 contains 75-80 characters in tag <400> and
E 310	Invalid sequence type in <212> in SEQID: (0)

Input Set:

Output Set:

Started: 2016-06-21 16:17:24.254
Finished: 2016-06-21 16:17:28.939
Elapsed: 0 hr(s) 0 min(s) 4 sec(s) 685 ms
Total Warnings: 2869
Total Errors: 78
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code	Error Description
E 248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E 202	Invalid input format; Value must be an integer in <211> in SEQID
E 248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header
W 402	Undefined organism found in <213> in SEQ ID (0)
E 444	Invalid line termination: on line 59 and tag: <400> line length validation was not performed
W 446	The current line: 61 contains 75-80 characters in tag <400> and
E 310	Invalid sequence type in <212> in SEQID: (0)
E 248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E 202	Invalid input format; Value must be an integer in <211> in SEQID
E 248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header
W 402	Undefined organism found in <213> in SEQ ID (0)
E 444	Invalid line termination: on line 71 and tag: <400> - line length validation was not performed
W 446	The current line: 73 contains 75-80 characters in tag <400> and
E 310	Invalid sequence type in <212> in SEQID: (0)
E 248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E 202	Invalid input format; Value must be an integer in <211> in SEQID
E 248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header
W 402	Undefined organism found in <213> in SEQ ID (0)
E 444	Invalid line termination: on line 83 and tag: <400> - line

Input Set:

Output Set:

Started: 2016-06-21 16:17:24.254
Finished: 2016-06-21 16:17:28.939
Elapsed: 0 hr(s) 0 min(s) 4 sec(s) 685 ms
Total Warnings: 2869
Total Errors: 78
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code		Error Description
		not performed
E	310	Invalid sequence type in <212> in SEQID: (0)
E	248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E	202	Invalid input format; Value must be an integer in <211> in SEQID
E	248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header
W	402	Undefined organism found in <213> in SEQ ID (0)
E	444	Invalid line termination: on line 95 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 97 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 99 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 101 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 103 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 105 and tag: <400> - line length validation was not performed
E	444	Invalid line termination: on line 107 and tag: <400> - line length validation was not performed This error has occured more than 20 times, will not be displayed
E	310	Invalid sequence type in <212> in SEQID: (0)
E	248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E	202	Invalid input format; Value must be an integer in <211> in SEQID
E	248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header

Input Set:

Output Set:

Started: 2016-06-21 16:17:24.254
Finished: 2016-06-21 16:17:28.939
Elapsed: 0 hr(s) 0 min(s) 4 sec(s) 685 ms
Total Warnings: 2869
Total Errors: 78
No. of SeqIDs Defined: 0
Actual SeqID Count: 0

Error code		Error Description
W	402	Undefined organism found in <213> in SEQ ID (0)
W	446	The current line: 149 contains 75-80 characters in tag <400> and Sequence ID (0)
E	310	Invalid sequence type in <212> in SEQID: (0)
E	248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E	202	Invalid input format; Value must be an integer in <211> in SEQID
E	248	Order Sequence Error <211> -> <213>; Expected Mandatory Tag: <212> in Header
W	402	Undefined organism found in <213> in SEQ ID (0)
E	310	Invalid sequence type in <212> in SEQID: (0)
E	248	Order Sequence Error <212> -> <211>; Expected Mandatory Tag: <213> in Header
E	202	Invalid input format; Value must be an integer in <211> in SEQID
E	248	Order Sequence Error <211> -> ; Expected Mandatory Tag: <212> in

Organization Applicant
 [illegible]
      Street : 4110 N. Freeway Blvd.
      City : Sacramento
      State : CA
      Country : US
      PostalCode : 95834
      PhoneNumber : 916-921-6145
      FaxNumber : 916-921-5611
      EmailAddress :
<110> OrganizationName : Ventria Bioscience

Application Project

<120> Title : CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
<130> AppFileReference : VBS0002-201-US
<140> CurrentApplNumber :
<141> CurrentFilingDate : ____-__-__

Earlier Applications

<150> PriorApplNumber : 61/154,204
<151> PriorFilingDate : 2009-02-20

Sequence

<213> OrganismName : Homo sapiens
<400> PreSequenceString :
MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF 60

EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP 120

ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF 180

FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV 240

ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK 300

ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR 360

RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE 420

QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV 480

LNQLCVLHEK TPVSDRVTKC CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL 540

SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV 600

AASQAALGL 609
<213> Type : PRT
<211> Length : 609
      SequenceName : SEQ ID 1:
      SequenceDescription :

Sequence

<213> OrganismName : Synthetic

<400> PreSequenceString :
ATAGDTHLGG EDFDNRVVPG PADKSPMIVV TYKGEEKNAV ITVPAYFNDS QRIINEPTAA 60

AIAYGLDKE 69
<212> Type : PRT
<211> Length : 69
SequenceName : SEQ ID 2:
SequenceDescription :

Sequence

<213> OrganismName : Synthetic
<400> PreSequenceString :
NQAAVIPERN GHVEIIANDQ GNRIVIKDGK PYIQVKIINE PTAAAIAYGL DKKKLGTVIG 60

IDLGTTYSCV GVYKVEIESL FDGTDSFSEP LTR 93
<212> Type : PRT
<211> Length : 93
SequenceName : SEQ ID 3:
SequenceDescription :

Sequence

<213> OrganismName : Synthetic
<400> PreSequenceString :
NQADSVVYQT EKKQDITITG ASTLPKDEVE RDVVLLDVTP LSLSLGLETL GGVMTK 56

<212> Type : PRT
<211> Length : 56
SequenceName : SEQ ID 4:
SequenceDescription :

Sequence

<213> OrganismName : Oryza Sativa
<400> PreSequenceString :
MAGNKGEGPA IGIDLGTTYS CVGVWQHDRV EIIANDQGNR TTPSYVAFTD TERLIGDAAK 60

NQVANNPTNT VFDAKRLIGR RFSDPSVQAD MKMWPFKVVP GPADKPMIVV TYKGEEKRFS 120

AEEISSMVLT KMKEIAEAFL GTTIKNAVIT VPAYFNDSQR QATKDAGVIS GLNVMRIINE 180

PTAAAIAYGL DKKAAGTGEK NVLIFDLGGG TFDVSILTIE EGIFEVKATA GDTHLGGEDF 240

DNRMVNHFVQ EFKRKHKFDI TGNPRALRRL RTACERAKRT LSSTAQTTIE IESLYEGIDF 300

YATITRARFE ELNMDLFRPC MEPVEKCLRD AKMDKAQIHD VVLVGGSTRI PKVQQLLQDF 360

FNGKELCKSI NPDEAVAYGA AVQAAILSGE GNQRVQDLLL LDVTPLSLGL ETAGGVMTVL 420

IPRNTTIPTK KEQVFSTYSD NQPGVLIQVY EGERTRTKDN NLLGKFELTG IPPAPRGVPQ 480

INVTFDIDAN GILNVSAEDK TTGKKNKITI TNDKGRLSKE EIERMVQEAE KYKAEDEQVR 540

DKVEAKNALE NYAYNMRNTV KDEKIASKLP ADDKKKIEDA IEDAIKWLDG NQLAEADEFE 600

```
DKNKELESLC NPIISKNYQG GAGGPAGNDE DAPNGSAGTG GGSGAGPKIE EVD                663


<212> Type : PRT
<211> Length : 663
      SequenceName : SEQ ID 5:
      SequenceDescription :

Sequence
--------
<213> OrganismName : Oryza Sativa
<400> PreSequenceString :
MDRVRGSAFL LGVLLAGSLF AFSVAKEETK KLGTVIGIDL GTTYSCVGVY KNGHVEIIAN         60

DQGNRITPSW VAFTDSERLI GEAAKNQAAV NPERTIFDVK RDIGRKFEEK EVQRDMKLVP        120

YKIVNKIGKP YIQVKIKDGE NKVFSPEEVS AMILGKMKET AEAYLGKKIN DAVVTVPAYF        180

NDAQRQATKD AGVIAGLNVA RIINEPTAAA IAYGLDKKGG EKNILVFDLG GGTFDVSILT        240

IDNGVFEVLA TNGDTHLGGE DFDQRIMEYF IKLIKKKYSK DISKDNRALG KLRREAERAK        300

RALSNQHQVR VEIESLFDGT DFSEPLTRAR FEELNMDLFR KTMGPVKKAM DDAGLEKSQI        360

HEIVLVGGST RIPKVQQLLR DYFEGKEPNK GVNPDEAVAY GAAVQGSILS GEGGDETKDI        420

LLLDVAPLTL GIETVGGVMT KLIPRNTVIP TKKSQVFTTY QDQQTTVSIQ VFEGERSMTK        480

DCRLLGKFDL SGIPAAPRGT PQIEVTFEVD ANGILNVKAE DKGTGKSEKI TITNEKGRLS        540

QEEIDRMVRE AEEFAEEDKK VKERIDARNQ LETYVYNMKN TVGDKDKLAD KLESEEKEKV        600

EEALEEALEW LDENQTAEKE EYEEKLKEVE AVCNPIISAV YQRTGGAPGG RRRGRLDDEH        660

DEL                                                                      663
<212> Type : PRT
<211> Length : 663
      SequenceName : SEQ ID 6:
      SequenceDescription :

Sequence
 --    -
<213> OrganismName : Oryza Sativa
<400> PreSequenceString :
MACFTCQLGA MACGAAPSTS PIAARRGGQL PVGRKPAAAC VQNRVPRAGR ARGVAMPVAC         60

EPVVGIDLGT TNSAVAAMEG GKPTVITNAE GQRTTPSVVA YTKGGERLVG QIAKRQAVVN        120

PENTFFSVKR FIGRKMAEVD DEAKQVSYHV VRDDNGNVKL DCPAIGKQFA AEEISAQVLR        180

KLVDDASKFL NDKITKAVVT VPAYFNDSQR TATKDAGRIA GLEVLRIINE PTAASLAYGF        240

EKKNNETILV FDLGGGTFDV SVLEVGDGVF EVLSTSGDTH LGGDDFDKFY FCWVFYFGAN        300

THETPKVVDW LASNFKKDEG IDLLKDKQAL QRLTEAAENA KMELSTLSQT NISLPFITAT        360

ADGPKHIETT LSRAKFEELC SDLIDRLKIP VTNALRDAKL SVDNLDEVIL VGGSTRIPSV        420
```

```
QELVKKITGK DPNVTVNPDE VVSLGAAVQG GVLAGDVKDV VLLDVTPLSL GLETLGGVMT        480

KIIPRNTTLP TSKSEVFSTA ADGQTSVEIN VLQGEREFVR DNKSLGSFRL DGIPPAPRGV        540

PQIEVKFDID ANGILSVAAI DKGTGKKQDI TITGASTLPK DEVERMVEEA EKFAQEDKEK        600

RDAIDTKNQA DSVVYQTEKQ LKELGDKVPA PVKEKVDAKL NELMEAIAGG STQSNKDAMA        660

ALNEEVMQIG QANYNQQPNA GAAGPTPGAD AGPTSSGGKG PNDGDVIDAD FTDSN             715


<212> Type : PRT
<211> Length : 715
      SequenceName : SEQ ID 7:
      SequenceDescription :
```

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	VBS0002-201CT-US

CONFIRMATION NO. 8304

72659
GLOBAL PATENT GROUP - VBS
1005 North Warson Road
Suite 404
ST. LOUIS, MO 63132

FORMALITIES LETTER

OC000000084097365

Date Mailed: 07/01/2016

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee must be submitted.
- The application examination fee must be submitted.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - The application contains drawings and the specification contains a brief description of the drawings. However, the specification does not contain a brief description of the several views of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(7) and/or a drawing(s) has not been labeled in accordance with 37 CFR 1.84(u)(1). If each figure is not labeled "Fig." with a consecutive Arabic numeral (1, 2, etc.) or an Arabic numeral and capital letter in the English alphabet (A, B, etc.), then the drawing(s) must be relabeled in accordance with 37 CFR 1.84(u)(1). In addition, if the brief description of the several views of the drawings does not refer to the figure(s) as properly labeled, then the specification must be amended to correspond to the figure(s) as properly labeled and a substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required.
- A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer reedable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing." Applicant must provide a substitute computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper or compact disc) sequence listing and, where applicable, includes no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). Refer to attachment or PAIR document dated 6/28/16.

For questions regarding compliance to these requirements, please contact:

- **For Rules interpretation, call (571) 272-2510**
- **For CRF submission help, call (571) 272-2510**
- **For PatentIn software program support technical assistance, call (866)217-9197.**
- **Send e-mail correspondence for PatentIn software program help to ebc@uspto.gov**
- **Patent software is available at http://www.uspto.gov/patents/resources/tools/checker/patentinrel.jsp .**

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Additional claim fees of **$ 3780** as a small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. Small entity discount is in effect. If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **70** basic filing fee.
- $ **200** for **17** electronically equivalent pages in excess of **100** application size fee.
- $ **70** surcharge.
- $ **300** search fee.
- $ **360** examination fee.
- $ **630** for **3** independent claims over 3.
- $ **2760** for **69** total claims over 20.
- $ **390** for multiple dependent claim surcharge
- $(**0**) previous unapplied payment amount.
- $ **4780** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Steven Clyde Pettit
  Mary Ann Michelle Fernandez Santos
  Ning Huang

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/kgebremichael/

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/188,478

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b) or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (l) or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p) or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	89 minus 20 =	* 69	x 40 =	2760	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	6 minus 3 =	* 3	x 210 =	630			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			200			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				390			
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL	4710		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15188478	

Applicant(s): Steven Pettit

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT		*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend		Indep	Depend	Indep	Depend	Indep	Depend
1	1						51						
2	1						52						
3	1						53						
4	1						54						
5	1						55						
6		2					56						
7		5					57						
8		5					58						
9		5					59						
10		5					60						
11		5					61						
12		1					62						
13		5					63						
14		5					64						
15		5					65						
16		5					66						
17		5					67						
18		5					68						
19		5					69						
20		5					70						
21		5					71						
22		(5)					72						
23	1						73						
24		1					74						
25		1					75						
26		1					76						
27		1					77						
28		1					78						
29		1					79						
30		1					80						
31		1					81						
32		1					82						
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34							84						
35							85						
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38							88						
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41							91						
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43							93						
44							94						
45							95						
46							96						
47							97						
48							98						
49							99						
50							100						
Total Indep	6		0		0								
Total Depend	83	↵	0	↵	0	↵							
Total Claims	89		0		0								

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/188,478	06/21/2016	1653	0.00	VBS0002-201C1-US	32	6

CONFIRMATION NO. 8304

72659
GLOBAL PATENT GROUP - VBS
1005 North Warson Road
Suite 404
ST. LOUIS, MO 63132

FILING RECEIPT

OC000000084097384

Date Mailed: 07/01/2016

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Steven Clyde Pettit, Fort Collins, CO;
Mary Ann Michelle Fernandez Santos, Elk Grove, CA;
Ning Huang, Davis, CA;

Applicant(s)

Ventria BioScience Inc., Fort Collins, CO

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 12/708,462 02/18/2010
which claims benefit of 61/154,204 02/20/2009

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 06/30/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/188,478**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies. particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired betore the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation. and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

Docket No.: VBS0002-201-US *GLOBAL PATENT GROUP, LLC*

Declaration for U.S. Patent Application and Appointment of Attorney

As a below named inventor, I hereby declare that
My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

[ ] the specification of which is attached hereto unless the following is checked

was filed on 02/18/2010 as United States Application Number or PCT International Application Number 12/708,462 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, ' 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, ' 119 (a) - (d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed

				Priority Claimed	
(List prior foreign applications.)	(Number)	(Country)	((Month/Day/Year Filed)	___ Yes	___ No
	(Number)	(Country)	(Month/Day/Year Filed)	___ Yes	___ No
	(Number)	(Country)	(Month/Day/Year Filed)	___ Yes	___ No
	(Number)	(Country)	((Month/Day/Year Filed)	___ Yes	___ No

☐ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, ' 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, ' 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, ' 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(List Prior U.S. Applications)	61/154,204 (Appln. Serial No.)	02/20/2009 (Filing Date)	Expired (Status: Patented, Pending, Abandoned)
	(Appln. Serial No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)
	(Appln. Serial No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)

I hereby appoint the firm of Global Patent Group, Customer Number 72659, including as principal attorneys and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Dennis A. Bennett, Reg. No. 34,547

Please direct all communications to the following address

associated with customer no. 72659

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, ' 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor (given name, family name)	Steven Clyde Pettit
Inventor's signature	Steven Clyde Pettit Date June, 17, 2010
Residence	Fort Collins, CO Citizenship United States
Post Office Address	~~2120 Milestone Drive, Suite 102, Fort Collins, CO 80525~~ 320 E. Vine Dr, Fort Collins, CO 80524
Full name of second inventor (given name, family name)	Mary Ann Michelle Fernandez Santos
Inventor's signature	Date
Residence	Elk Grove, CA Citizenship United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525

[ ] See following pages for additional joint inventors

Full name of second inventor (given name, family name)	Ning Huang
Inventor's signature	Date
Residence	Davis, CA Citizenship United States
Post Office Address	2120 Milestone Drive Suite 102, Fort Collins CO 80525

[ ] See following pages for additional joint inventors

Please direct all communications to the following address:

Associated with Customer no. 72659

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, ' 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor (given name, family name)	Steven Clyde Pettit
Inventor's signature	Date
Residence	Fort Collins, CO — Citizenship: United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525

Full name of second inventor (given name, family name)	Mary Ann Michelle Fernandez Santos
Inventor's signature	Mary Ann Michelle Fernandez Santos — Date: 12/16/10
Residence	Elk Grove, CA — Citizenship: United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525

| | See following pages for additional joint inventors

Full name of second inventor (given name, family name)	Ning Huang
Inventor's signature	Date
Residence	Davis, CA — Citizenship: United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525

| | See following pages for additional joint inventors

Please direct all communications to the following address:

[illegible]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, ' 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor (given name, family name)	Steven Clyde Pettit		
Inventor's signature		Date	
Residence	Fort Collins, CO	Citizenship	United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525		

Full name of second inventor (given name, family name)	Mary Ann Michelle Fernandez Santos		
Inventor's signature		Date	
Residence	Elk Grove, CA	Citizenship	United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525		

[ ] See following pages for additional joint inventors

Full name of second inventor (given name, family name)	Ning Huang		
Inventor's signature		Date	12/16/2010
Residence	Davis, CA	Citizenship	United States
Post Office Address	2120 Milestone Drive, Suite 102, Fort Collins, CO 80525		

[ ] See following pages for additional joint inventors

Electronic Patent Application Fee Transmittal					
Application Number:	15188478				
Filing Date:	21-Jun-2016				
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS				
First Named Inventor/Applicant Name:	Steven Clyde Perrit				
Filer:	Dennis A. Bennett				
Attorney Docket Number:	VBS0002-201C1-US				
Filed as Small Entity					
Filing Fees for Utility under 35 USC 111(a)					
Description		**Fee Code**	**Quantity**	**Amount**	**Sub-Total in USD($)**
Basic Filing:					
Pages:					
Claims:					
Miscellaneous-Filing:					
LATE FILING FEE FOR OATH OR DECLARATION		2051	1	70	70
Petition:					
Patent-Appeals-and-Interference:					
Post-Allowance-and-Post-Issuance:					

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	70

Electronic Acknowledgement Receipt	
EFS ID:	27782969
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	72659
Filer:	Dennis A. Bennett
Filer Authorized By:	
Attorney Docket Number:	VBS0002-201C1-US
Receipt Date:	13-DEC-2016
Filing Date:	21-JUN-2016
Time Stamp:	17:27:49
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$70
RAM confirmation Number	121416INTEFSW00005567504297
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	VBS0002_ExecDeclaration.pdf	132756	no	4
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	29893	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	162649		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	VBS0002-201CT-US

CONFIRMATION NO. 8304

72659
GLOBAL PATENT GROUP - VBS
17014 NEW COLLEGE AVENUE
SUITE 201
WILDWOOD, MO 63040

FORMALITIES LETTER

OC000000087922558

Date Mailed: 12/16/2016

NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

Filing Date Granted

The U.S. Patent and Trademark Office has received your reply on 12/13/2016 to the Notice to File Missing Parts (Notice) mailed 07/01/2016 and it has been entered into the nonprovisional application. The reply, however, does not include the following items required in the Notice. A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application. Replies should be mailed to: Mail Stop Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria VA 22313-1450.

Applicant is given **TWO MONTHS** from the date of the Notice to File Missing Parts (Notice) mailed 07/01/2016 within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee was not received.
- The application examination fee was not received.

The application is informal since it does not comply with the regulations for the reason(s) indicated below

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
  - The application contains drawings and the specification contains a brief description of the drawings. However, the specification does not contain a brief description of the several views of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(7) and/or a drawing(s) has not been labeled in accordance with 37 CFR 1.84(u)(1). If each figure is not labeled "Fig." with a consecutive Arabic numeral (1, 2, etc.) or an Arabic numeral and capital letter in the English alphabet (A, B, etc.), then the drawing(s) must be relabeled in accordance with 37 CFR 1.84(u)(1). In addition, if the brief description of the several views of the drawings does not refer to the figure(s) as properly labeled, then the specification must be amended to correspond to the figure(s) as properly labeled and a substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required.
- A copy of the "Sequence Listing" in computer readable form has been submitted. However, the content of the computer readable form does not comply with the requirements of 37 CFR 1.822 and/or 1.823, as indicated on the attached copy of the marked -up "Raw Sequence Listing." Applicant must provide a substitute computer readable form (CRF) copy of the "Sequence Listing" and a statement that the content of the sequence listing information recorded in computer readable form is identical to the written (on paper

or compact disc) sequence listing and, where applicable, includes no new matter. as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b), or 1.825(d). Refer to attachment or PAIR document dated 6/28/16.

For questions regarding compliance to these requirements, please contact:

- **For Rules interpretation, call (571) 272-2510**
- **For CRF submission help, call (571) 272-2510**
- **For PatentIn software program support technical assistance, call (866)217-9197.**
- **Send e-mail correspondence for PatentIn software program help to ebc@uspto.gov**
- **Patent software is available at http://www.uspto.gov/patents/resources/tools/checker/patentinrel.jsp .**

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

- Additional claim fees of $ **3780** as a small entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of the Notice to avoid abandonment is/are itemized below. Small entity discount is in effect. If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **70** basic filing fee.
- $ **200** for **17** electronically equivalent pages in excess of **100** application size fee.
- $ **300** search fee.
- $ **360** examination fee.
- $ **630** for **3** independent claims over 3.
- $ **2760** for **69** total claims over 20.
- $ **390** for multiple dependent claim surcharge
- $(**0**) previous unapplied payment amount.
- $ **4710** TOTAL FEE BALANCE DUE.

Items Required To Avoid Processing Delays:

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

A new inventor's oath or declaration that identifies this application (e.g., by Application Number and filing date) is required. The inventor's oath or declaration does not comply with 37 CFR 1.63 in that it:

- does not state that the above-identified application was made or authorized to be made by the person executing the oath or declaration.

  Steven Clyde Pettit
  Mary Ann Michelle Fernandez Santos
  Ning Huang

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/tha/

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/188,478

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b) or (c))	N/A	N/A	N/A	70		N/A	
SEARCH FEE (37 CFR 1.16(k), (l) or (m))	N/A	N/A	N/A	300		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p) or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	89 minus 20 =	* 69	x 40 =	2760	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	6 minus 3 =	* 3	x 210 =	630			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			200			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				390			
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL	4710		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	VBS0002-201CT-US

CONFIRMATION NO. 8304

72659
GLOBAL PATENT GROUP - VBS
17014 NEW COLLEGE AVENUE
SUITE 201
WILDWOOD, MO 63040

ABANDONMENT/TERMINATION LETTER

OC000000089570170

Date Mailed: 03/02/2017

NOTICE OF ABANDONMENT UNDER 37 CFR 1.53 (f) OR (g)

The above-identified application is abandoned for failure to timely or properly reply to the Notice to File Missing Parts (Notice) mailed on 07/01/2016.

- The reply received on 12/13/2016 was insufficient. The reply did not include:
  - The full amount of the basic filing fee under 37 CFR 1.16(a), (b), (c), or (d). *Note: A nonprovisional application may not be relied on for benefits under 35 U.S.C. 120 and 37 CFR 1.78* ***unless the basic filing fee is paid within the pendency of the application*** *. A provisional application may not be relied on for benefits under 35 U.S.C. 119(e) and 37 CFR 1.78* ***unless the basic filing fee is paid within the time period set forth in 37 CFR 1.53(g).***
  - The full amount of the search and examination fees required under 37 CFR 1.16(k)-(r).
  - Replacement drawings as required.

If a complete reply to the notice was previously filed by applicant within the time period set forth in the notice, applicant may request for reconsideration of the holding of abandonment within 2 months from the mailing of this notice of abandonment by filing a petition to withdraw the holding of abandonment under 37 CFR 1.181(a) No petition fee is required. The petition must be accompanied by a true copy of the originally filed reply and the item(s) identified in one of the following:

1. A properly itemized date-stamped postcard receipt (see MPEP § 503);

2. If the originally filed reply included a certificate of mailing or transmission in compliance with 37 CFR 1.8(a), a copy of the certificate of mailing or transmission and a statement in compliance with 37 CFR 1.8(b) (see MPEP § 512); or

3. If the reply was filed via "Express Mail", (now "Priority Mail Express"), a submission satisfying the requirements of 37 CFR 1.10(e) including, for example, a copy of the mailing label showing the "date-in" (or "date accepted") (see MPEP § 513).

If applicant did not previously file complete reply within the the time period set forth in the notice, applicant may file a petition to revive the application under 37 CFR 1.137.

Under 37 CFR 1.137, a petition requesting that the application be revived on the grounds of UNINTENTIONAL DELAY must be filed promptly after applicant becomes aware of the abandonment and such petition must be accompanied by: (1) the reply required to the outstanding Office action or notice, unless previously filed; (2) the petition fee set forth in 37 CFR 1.17(m); (3) a terminal disclaimer (and fee set forth in 37 CFR 1.20(d)) if required by 37 CFR 1.137(d); and (4) a statement that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition was unintentional. See MPEP 711.03(c) and Form PTO/SB/64.

Any questions concerning petitions to revive should be directed to the Office of Petitions at (571) 272-3282. Petitions should be mailed to Mail Stop Petitions, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

A copy of this notice MUST be returned with the reply.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/zmoguss/

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15188478 Document Date: 03/16/2017

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

Doc Code: PET.OP
Document Description: Petition for Review by the Office of Petitions

PTO/SB/64 (12-13)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR REVIVAL OF AN APPLICATION FOR PATENT ABANDONED UNINTENTIONALLY UNDER 37 CFR 1.137(a)

Page 1 of 2

Docket Number (Optional)
VBS0002-201C1-US

First named inventor: Steven Clyde Pettit

Application No.: 15/188,478 Art Unit: 1653

Filed: June 21, 2016 Examiner: To Be Assigned

Title: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

Attention: Office of Petitions
Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
FAX (571) 273-8300

NOTE: If information or assistance is needed in completing this form, please contact the Office of Petitions at (571) 272-3282.

The above identified application became abandoned for failure to file a timely and proper reply to a notice or action by the United States Patent and Trademark Office. The date of abandonment is the day after the expiration date of the period set for reply in the Office notice or action plus any extensions of time actually obtained.

APPLICANT HEREBY PETITIONS FOR REVIVAL OF THIS APPLICATION.

NOTE. A grantable petition requires the following items:

(1) Petition fee;
(2) Reply and/or issue fee;
(3) Terminal disclaimer with disclaimer fee – required for all utility and plant applications filed before June 8, 1995, and for all design applications; and
(4) Statement that the entire delay was unintentional.

1. **Petition fee**

☑ Small entity fee $ 850 (37 CFR 1.17(m)). Applicant asserts small entity status. See 37 CFR 1.27.

☐ Undiscounted fee $ _____ (37 CFR 1.17(m))

2. **Reply and/or fee**

A The reply and/or fee to the above noted Office notice or action in the form of

Response to Missing Parts, Replacement Drawings, Sequence Listing, and Fees (identify the type of reply):

☐ has been filed previously on _____.

☑ is enclosed herewith.

B The issue fee and publication fee (if applicable) of $ _____

☐ has been paid previously on _____.

☐ is enclosed herewith.

This collection of information is required by 37 CFR 1.137(a). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11, 1.14 and 41.6. This collection is estimated to take 1 hour to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Petition, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1 800 PTO 9199 and select option 2.

Doc Code: PET.OP
Document Description: Petition for Review by the Office of Petitions

PTO/SB/64 (12-13)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PETITION FOR REVIVAL OF AN APPLICATION FOR PATENT ABANDONED UNINTENTIONALLY UNDER 37 CFR 1.137(a)

3. **Terminal disclaimer with disclaimer fee**

☑ Since this utility/plant application was filed on or after June 8, 1995, no terminal disclaimer is required.

☐ A terminal disclaimer (and disclaimer fee (37 CFR 1.20(d)) of $ _____) disclaiming the required period of time is enclosed herewith (see PTO/SB/63).

4. **STATEMENT:** The entire delay in filing the required reply from the due date for the required reply until the filing of a grantable petition under 37 CFR 1.137(a) was unintentional. [NOTE: The United States Patent and Trademark Office may require additional information if there is a question as to whether either the abandonment or the delay in filing a petition under 37 CFR 1.137(a) was unintentional. (MPEP 711.03(c), subsections (III)(C) and (D)).]

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

/Dennis A. Bennett/	March 16, 2017
Signature	Date
Dennis A. Bennett	34,547
Typed or Printed Name	Registration Number, if applicable
17014 New College Avenue, Suite 201	314-812-8020
Address	Telephone Number
St. Louis, MO 63040	
Address	

Enclosures:

☑ Fee Payment

☑ Reply

☐ Terminal Disclaimer Form

☐ Additional sheet(s) containing statements establishing unintentional delay

☐ Other: _____

CERTIFICATE OF MAILING OR TRANSMISSION [37 CFR 1.8(a)]

I hereby certify that this correspondence is being:

☐ Deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to: Mail Stop Petition, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450.

☑ Transmitted by EFS-Web or facsimile on the date shown below to the United States Patent and Trademark Office at (571) 273-8300.

March 16, 2017	/Lauren Wilson/
Date	Signature
	Lauren Wilson
	Typed or printed name of person signing certificate

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2), (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent

The information provided by you in this form will be subject to the following routine uses

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal

Application Number:	15188478
Filing Date:	21-Jun-2016
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Perrit
Filer:	Dennis A. Bennett/Lauren Wilson
Attorney Docket Number:	VBS0002-201C1-US

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY FILING FEE (ELECTRONIC FILING)	4011	1	70	70
UTILITY SEARCH FEE	2111	1	300	300
UTILITY EXAMINATION FEE	2311	1	360	360
Pages:				
Claims:				
Miscellaneous-Filing:				
LATE FILING FEE FOR OATH OR DECLARATION	2051	1	70	70
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
PET. REVIVE ABANDON APP, DELAY PYMT-RESP	2453	1	850	850
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 5 months with $0 paid	2255	1	1500	1500
Miscellaneous:				
		Total in USD ($)		**3150**

Electronic Acknowledgement Receipt	
EFS ID:	28654939
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	72659
Filer:	Dennis A. Bennett/Lauren Wilson
Filer Authorized By:	Dennis A. Bennett
Attorney Docket Number:	VBS0002-201C1-US
Receipt Date:	16-MAR-2017
Filing Date:	21-JUN-2016
Time Stamp:	18:02:16
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$3150
RAM confirmation Number	031717INTEFSW00004622504297
Deposit Account	504297
Authorized User	Lauren Wilson
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		20170315_Response_to_NMP.pdf	33924	yes	6

	Multipart Description/PDF files in .zip description		
	Document Description	Start	End
	Applicant Response to Pre-Exam Formalities Notice	1	1
	Specification	2	3
	Claims	4	5
	Applicant Arguments/Remarks Made in an Amendment	6	6

Warnings:

Information:

2	Drawings-only black and white line drawings	20170315_Replacement_Figures.pdf	4321246	no	20

Warnings:

Information:

3	Petition for review by the Office of Petitions	20170316_Petition_for_Revival.pdf	238403	no	3

Warnings:

Information:

4	Sequence Listing (Text File)	20170315_Sequence_Listing.txt	25264	no	-

Warnings:

Information:

5	Fee Worksheet (SB06)	fee-info.pdf	40619 [illegible]	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	4659456		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of	:	
	:	
Pettit, Steven Clyde et al.	:	Confirmation No. 8304
	:	
U.S. Patent Application No. 15/188,478	:	Group Art Unit: 1653
	:	
Filed: 06-21-2016	:	Examiner: To Be Assigned

Title: **CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS**

RESPONSE TO NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION UNDER 37 CFR 1.53(b) AND PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This letter is in response to the Notice to File Missing Parts, issued by the Office on July 1, 2016, for which a two-month shortened statutory period for reply was specified which expired on September 1, 2016.

Applicants submit the required fees, a preliminary amendment, replacement drawings, and computer readable Sequence Listing as requested.

Authorization is hereby given to treat this and any future reply, which requires or might require a petition for an extension of time under 37 CFR § 1.136(a) for its timely submission or payment of fee, as incorporating a petition for extension of time for the appropriate length of time and an authorization to pay any required fees from Deposit Account No. 50-4297.

Amendments to the Specification begin on page 2.

Amendments to the Claims begin on page 4.

Remarks follow Amendments to the Claims.

Amendments to the Specification

Applicants request amendment of the specification as follows in accordance with 37 C.F.R. § 1.21. Please amend the following paragraphs:

On Page 1, please amend the paragraph directly under the heading CROSS-REFERENCE TO RELATED APPLICATIONS as follows:

This application is a continuation application of US patent application 12/708,462 filed February 18, 2010, which claims the benefit of priority of US provisional patent application No. 61/154,204, filed February 20, 2009, the entire contents of each ~~which~~ are incorporated herein by reference.

On Page 18, please amend the paragraphs under BRIEF DESCRIPTION OF THE FIGURES as follows:

Page 18 at Line 7:

~~**Figure 1**~~ **Figure 1A - Figure 1D** is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

Page 18 at Line 27:

~~**Figure 5**~~ **Figure 5A - Figure 5D** shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

Page 19 at Line 25:

Figure 10: ~~**(Figure 10A) Shows**~~ **Figure 10A shows** a comparison of the effects of yeast recombinant HSA (Cellprime), human plasma derived HSA, (Seracare) and plant recombinant HSA (Cellastim P0171) with respect to their ability to promote cell growth and viability. ~~**(Figure 10A)**~~. **Figure 10B** shows a comparison of the endotoxin levels in batches of albumin produced using the old (B000) and new processes (B0000C) for recombinant albumin production. **Figure**

10C shows a comparison of cell growth and viability of cells grown in the presence of the Cellastim HSA produced using the old (B000) and new processes (B0000C) for recombinant albumin production.

Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims

1-34. (Canceled)

35. (New) A supplement or complete medium comprising:

a recombinant albumin wherein said recombinant albumin is:

i) produced in a plant;

ii) has less than 1 EU of endotoxin / mg of albumin; and

iii) less than 2 % aggregated albumin; and

wherein said supplement or complete medium improves growth of a cell in cell culture medium comprising said supplement or complete medium.

36. (New) The supplement of claim 35, wherein said recombinant albumin comprises at least 0,01 % wt / wt of a heat shock protein.

37. (New) The supplement of claim 36, wherein said heat shock protein is a rice heat shock protein.

38. (New) The supplement of claim 36, wherein said heat shock protein is selected from said group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein.

39. (New) The supplement of claim 36, wherein said heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469.

40. (New) The supplement of claim 36, wherein said supplement comprises at least 0.04 % wt / wt HSP70.

41. (New) The supplement of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is lactoferrin.

42. (New) The supplement of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is transferrin.

43. (New) The supplement of claim 42, wherein said ratio of said transferrin to said recombinant albumin is 1 to 50, to 1 to 5000.

44. (New) The supplement of claim 43, wherein said ratio of said lactoferrin to said recombinant albumin is 1 to 3, to 1 to 0.33.

Remarks

Applicants request amendment of the specification as shown above, responsive to the Office's requirement in preparation for publication. Applicants consider these amendments to be technical and not substantive in nature, and intend that the amendments above maintain the same scope of description in the specification as before, without paragraph numbers which would be confusing upon publication. No new matter has been added.

Claims 1-34 are canceled. Claims 35-44 are new and ready for examination.

Substitution of the drawings with the replacement sheets are filed concurrently herewith. No new matter is introduced by the replacement sheets.

Applicants' undersigned representative hereby states that, to the best of his knowledge, the sequence listing filed herewith in electronic form does not go beyond the disclosure in the US Application Serial No.: 14/687,601 as filed. In addition, no new matter has been added and the content of the sequence listing information provided herein is identical to the written sequence listing as previously submitted and required by 37 CFR 1.821(c), 1.821(f), 1.821(g), 1.825(b), or 1.825(d).

Early and favorable action on the merits is earnestly solicited.

The Examiner is encouraged to contact the Applicants' undersigned attorney to discuss this matter if any questions should arise upon further examination of the pending claims.

Respectfully submitted,

March 15, 2017
Date

/Dennis A. Bennett/
Dennis A. Bennett, Reg. No. 34,547
Attorney for Applicants

Global Patent Group, LLC
17014 New College Avenue, Suite 201
St. Louis, MO 63040
TEL: 314-812-8020

FIG. 1A

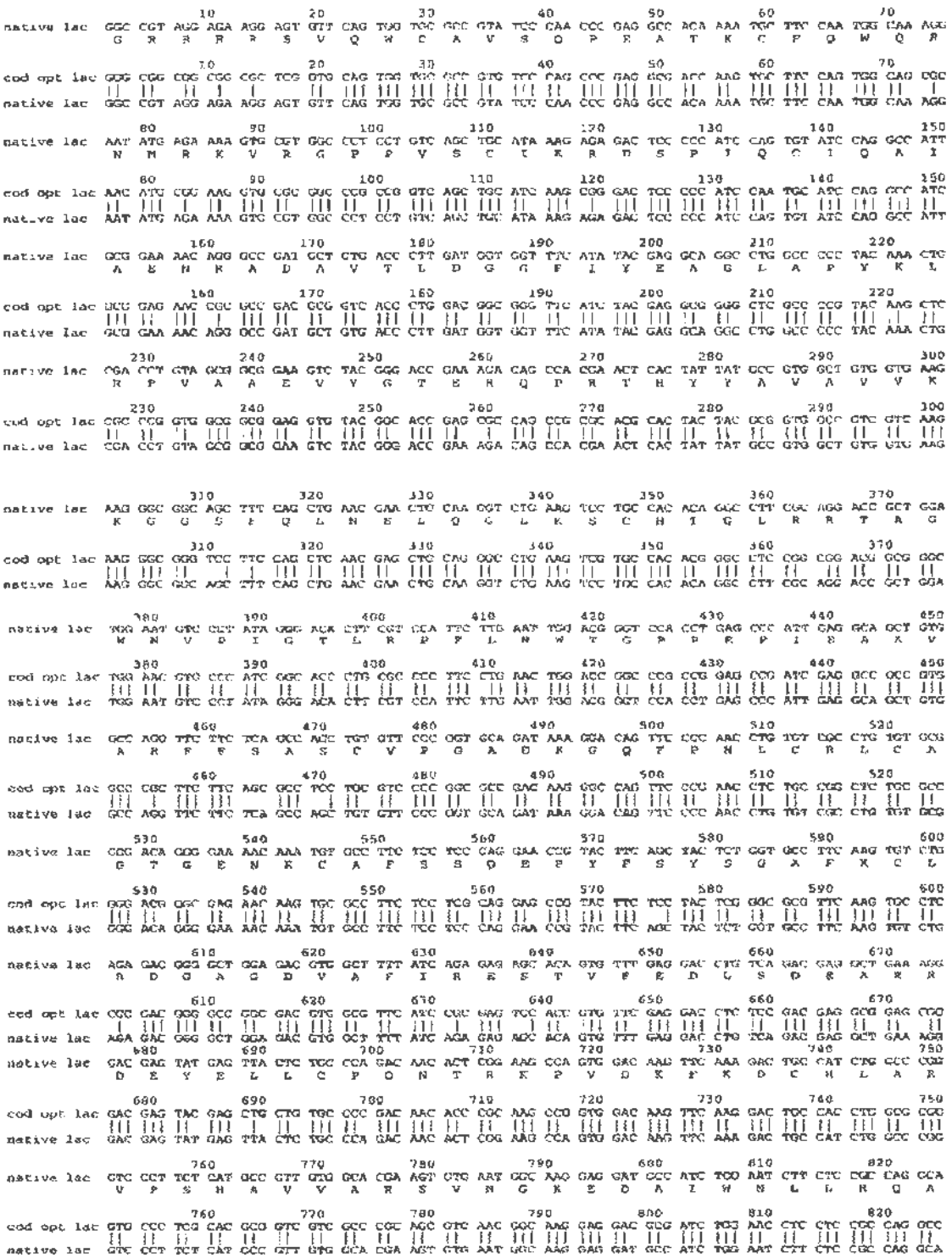


FIG. 1B

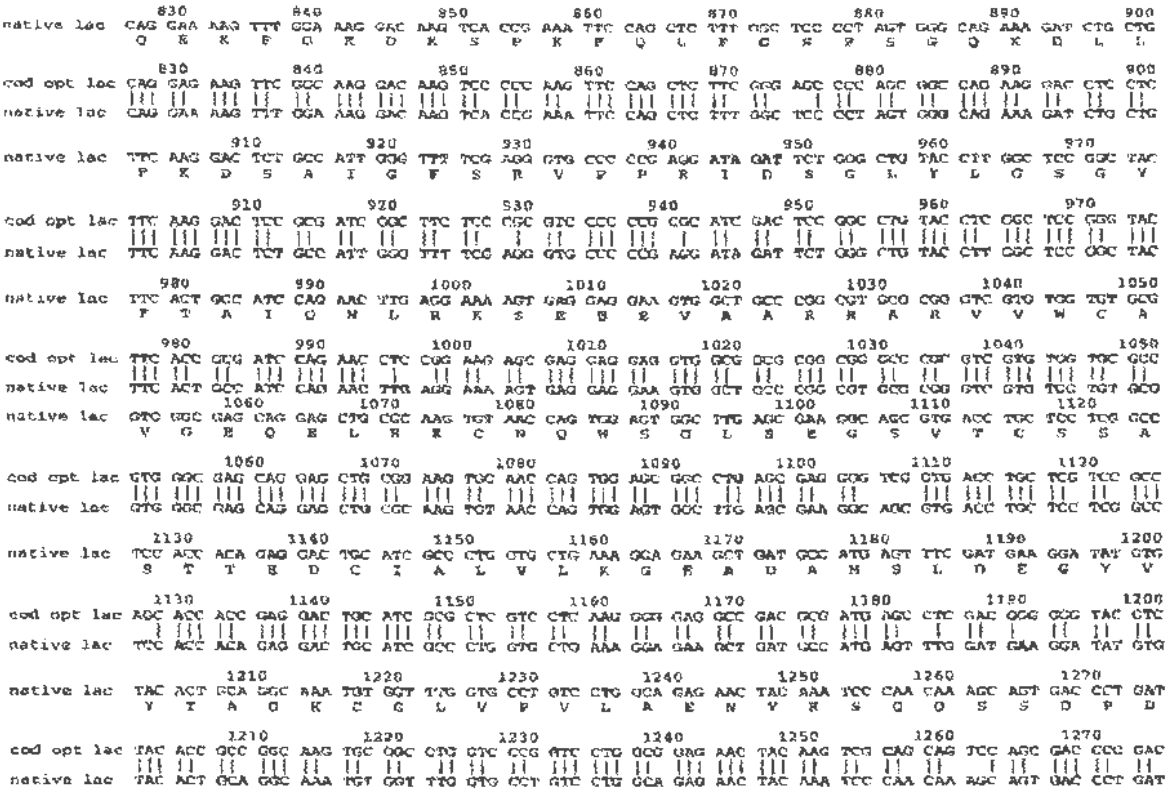


FIG. 1C

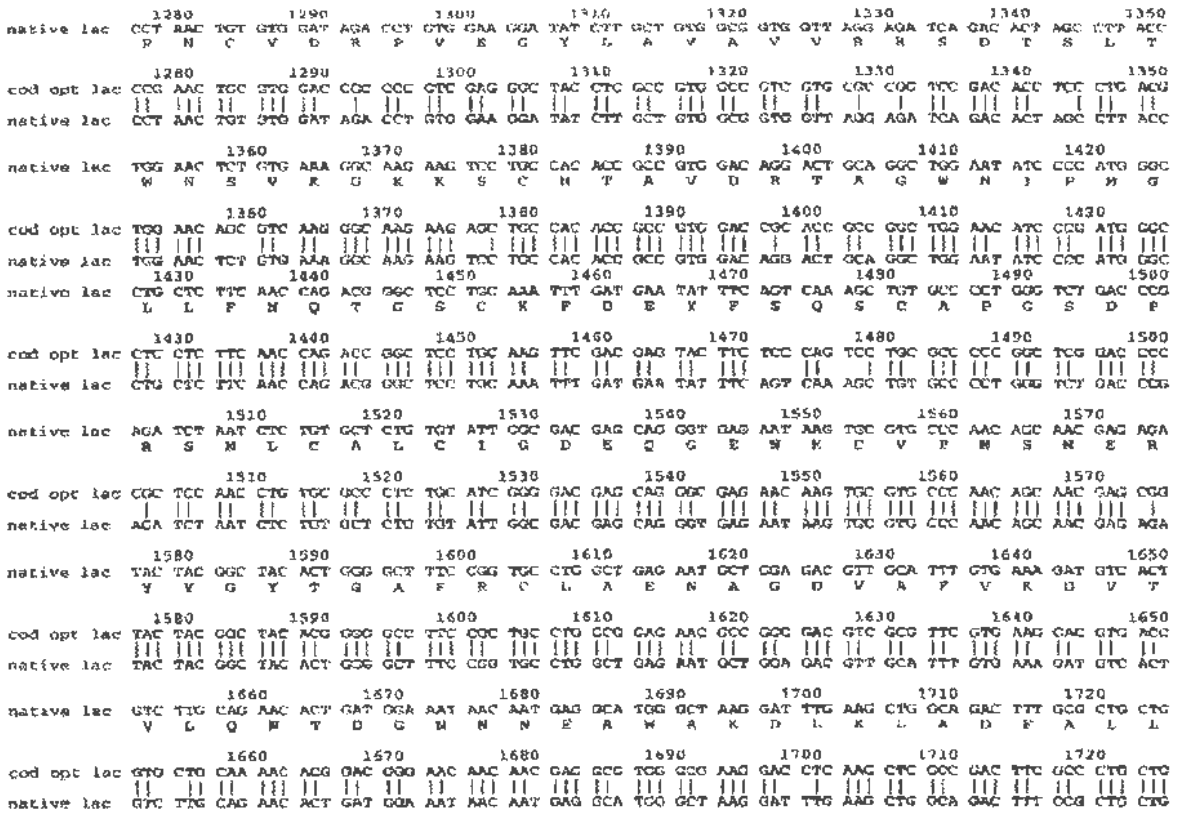


FIG 1D

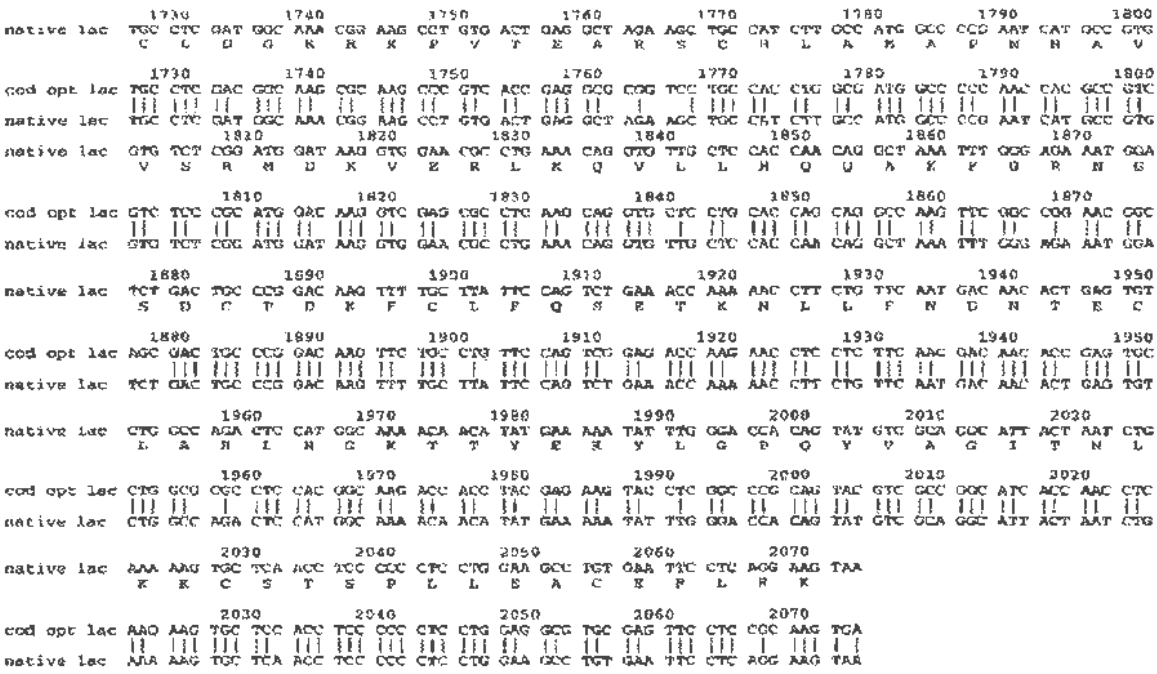

native lac
cod opt lac
2079 = Total number of nucleotides
467 = Number of nucleotides changed
693 = Total Number of codons
413 = Number of codons changed

FIG. 2

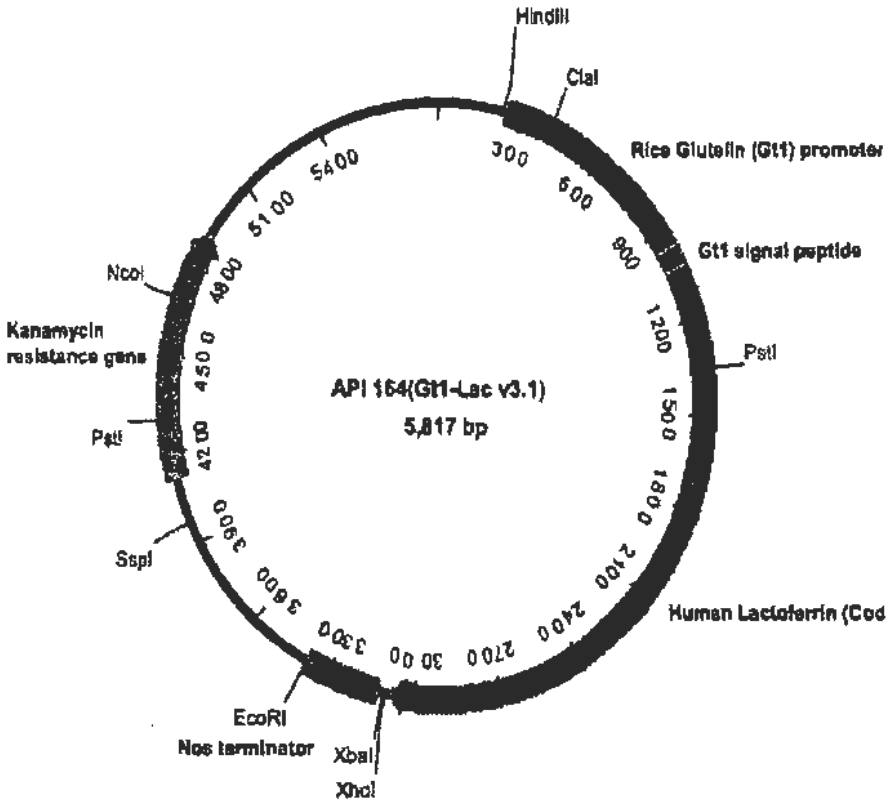

HindIII
ClaI
Rice Glutelin (Gt1) promoter
Gt1 signal peptide
PstI
Human Lactoferrin (Cod
XhoI
XbaI
EcoRI
Nos terminator
SspI
PstI
NcoI
Kanamycin resistance gene
API 164(Gt1-Lac v3.1)
5,817 bp
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
3600
3900
4200
4500
4800
5100
5400

FIG. 3

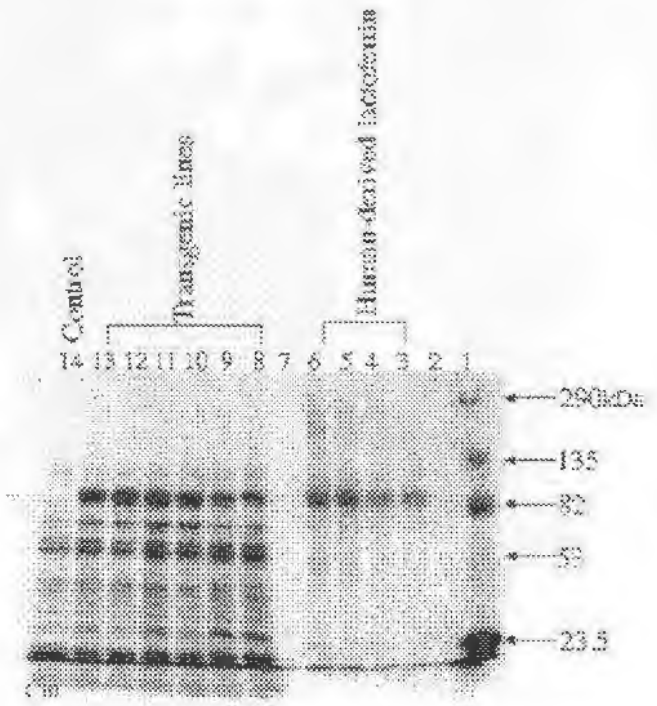

Control
Transgenic Lines
Human-derived Isolate
14 13 12 11 10 9 8 7 6 5 4 3 2 1
135
82
49
23.5

FIG. 4

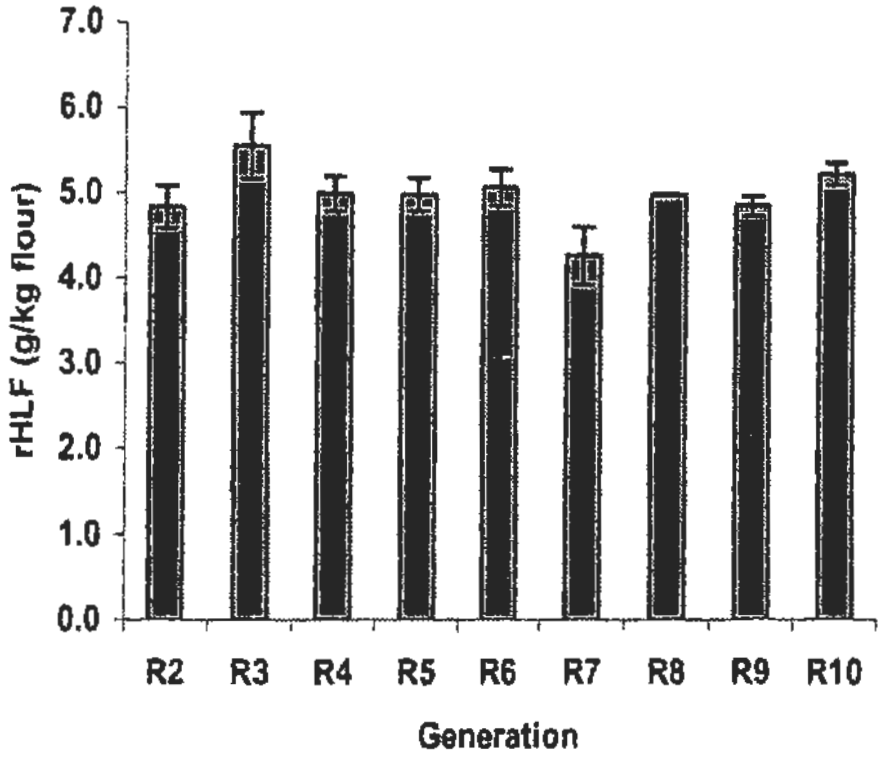

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
rHLF (g/Kg flour)
R2
R3
R4
R5
R6
R7
R8
R9
R10
Generation

FIG 5A

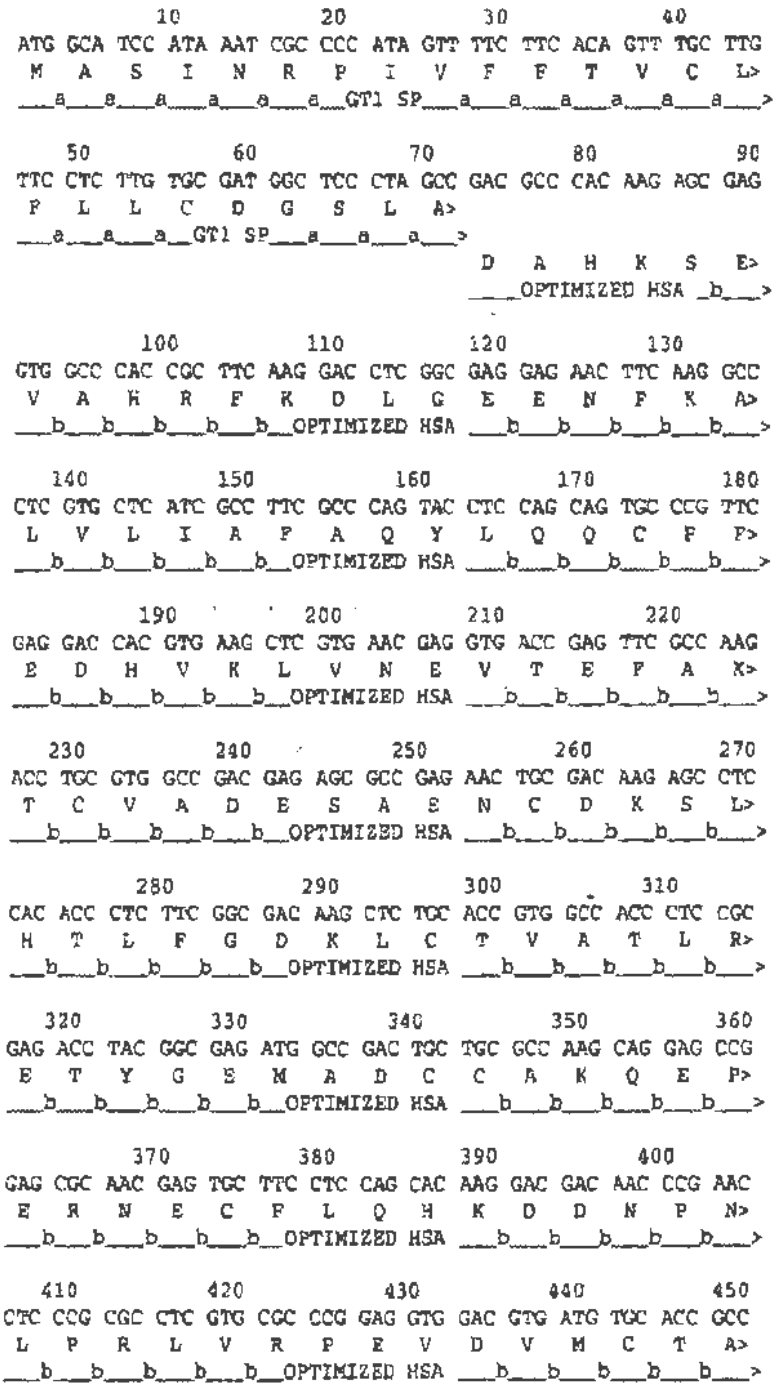

10 20 30 40
ATG GCA TCC ATA AAT CGC CCC ATA GTT TTC TTC ACA GTT TGC TTG
M A S I N R P I V F F T V C L>
a GT1 SP
50 60 70 80 90
TTC CTC TTG TGC GAT GGC TCC CTA GCC GAC GCC CAC AAG AGC GAG
F L L C D G S L A>
GT1 SP
D A H K S E>
OPTIMIZED HSA
100 110 120 130
GTG GCC CAC CGC TTC AAG GAC CTC GGC GAG GAG AAC TTC AAG GCC
V A H R F K D L G E E N F K A>
OPTIMIZED HSA
140 150 160 170 180
CTC GTG CTC ATC GCC TTC GCC CAG TAC CTC CAG CAG TGC CCG TTC
L V L I A F A Q Y L Q Q C P F>
OPTIMIZED HSA
190 200 210 220
GAG GAC CAC GTG AAG CTC GTG AAC GAG GTG ACC GAG TTC GCC AAG
E D H V K L V N E V T E F A K>
OPTIMIZED HSA
230 240 250 260 270
ACC TGC GTG GCC GAC GAG AGC GCC GAG AAC TGC GAC AAG AGC CTC
T C V A D E S A E N C D K S L>
OPTIMIZED HSA
280 290 300 310
CAC ACC CTC TTC GGC GAC AAG CTC TGC ACC GTG GCC ACC CTC CGC
H T L F G D K L C T V A T L R>
OPTIMIZED HSA
320 330 340 350 360
GAG ACC TAC GGC GAG ATG GCC GAC TGC TGC GCC AAG CAG GAG CCG
E T Y G E M A D C C A K Q E P>
OPTIMIZED HSA
370 380 390 400
GAG CGC AAC GAG TGC TTC CTC CAG CAC AAG GAC GAC AAC CCG AAC
E R N E C F L Q H K D D N P N>
OPTIMIZED HSA
410 420 430 440 450
CTC CCG CGC CTC GTG CGC CCG GAG GTG GAC GTG ATG TGC ACC GCC
L P R L V R P E V D V M C T A>
OPTIMIZED HSA

FIG 5B

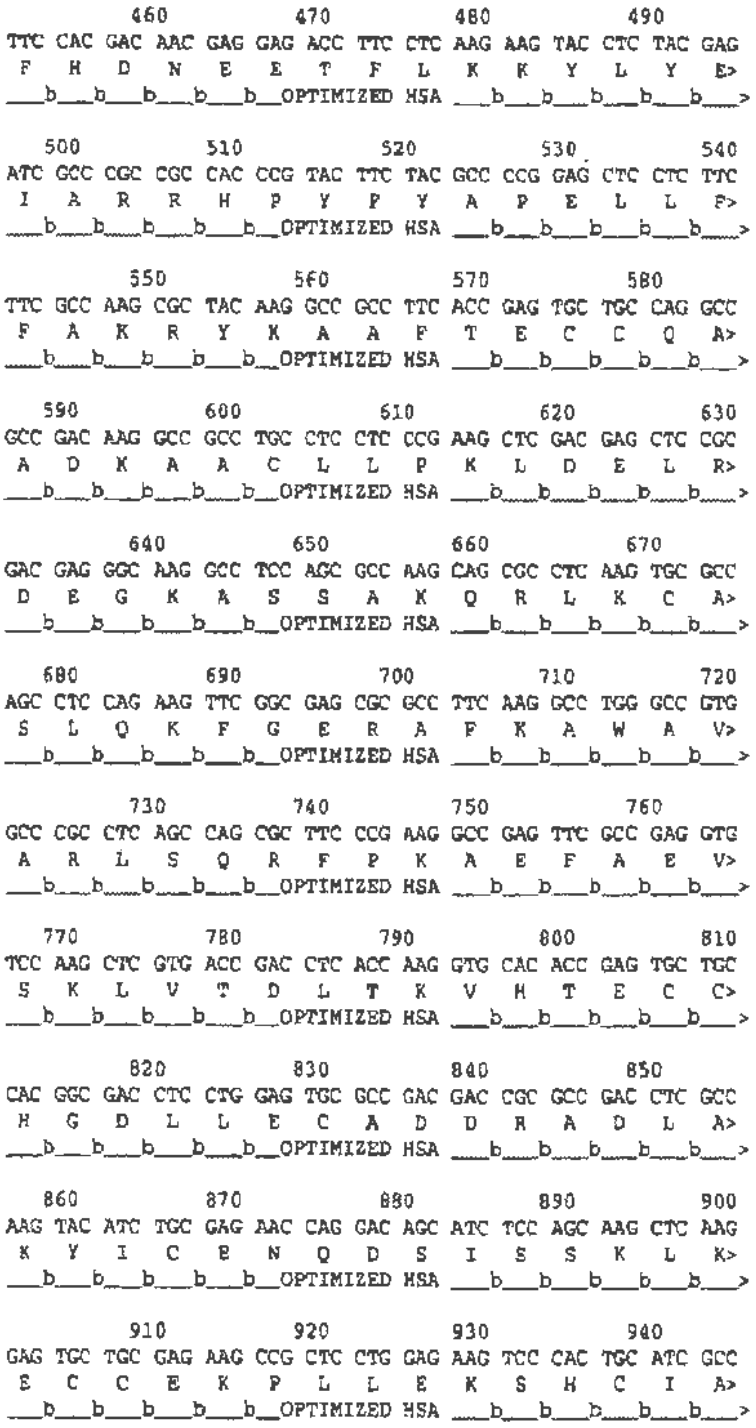

460 470 480 490
TTC CAC GAC AAC GAG GAG ACC TTC CTC AAG AAG TAC CTC TAC GAG
F H D N E E T F L K K Y L Y E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
500 510 520 530 540
ATC GCC CGC CGC CAC CCG TAC TTC TAC GCC CCG GAG CTC CTC TTC
I A R R H P Y P Y A P E L L F>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
550 560 570 580
TTC GCC AAG CGC TAC AAG GCC GCC TTC ACC GAG TGC TGC CAG GCC
F A K R Y K A A F T E C C Q A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
590 600 610 620 630
GCC GAC AAG GCC GCC TGC CTC CTC CCG AAG CTC GAC GAG CTC CGC
A D K A A C L L P K L D E L R>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
640 650 660 670
GAC GAG GGC AAG GCC TCC AGC GCC AAG CAG CGC CTC AAG TGC GCC
D E G K A S S A K Q R L K C A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
680 690 700 710 720
AGC CTC CAG AAG TTC GGC GAG CGC GCC TTC AAG GCC TGG GCC GTG
S L Q K F G E R A F K A W A V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
730 740 750 760
GCC CGC CTC AGC CAG CGC TTC CCG AAG GCC GAG TTC GCC GAG GTG
A R L S Q R F P K A E F A E V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
770 780 790 800 810
TCC AAG CTC GTG ACC GAC CTC ACC AAG GTG CAC ACC GAG TGC TGC
S K L V T D L T K V H T E C C>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
820 830 840 850
CAC GGC GAC CTC CTG GAG TGC GCC GAC GAC CGC GCC GAC CTC GCC
H G D L L E C A D D R A D L A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
860 870 880 890 900
AAG TAC ATC TGC GAG AAC CAG GAC AGC ATC TCC AGC AAG CTC AAG
K Y I C E N Q D S I S S K L K>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
910 920 930 940
GAG TGC TGC GAG AAG CCG CTC CTG GAG AAG TCC CAC TGC ATC GCC
E C C E K P L L E K S H C I A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

FIG 5C

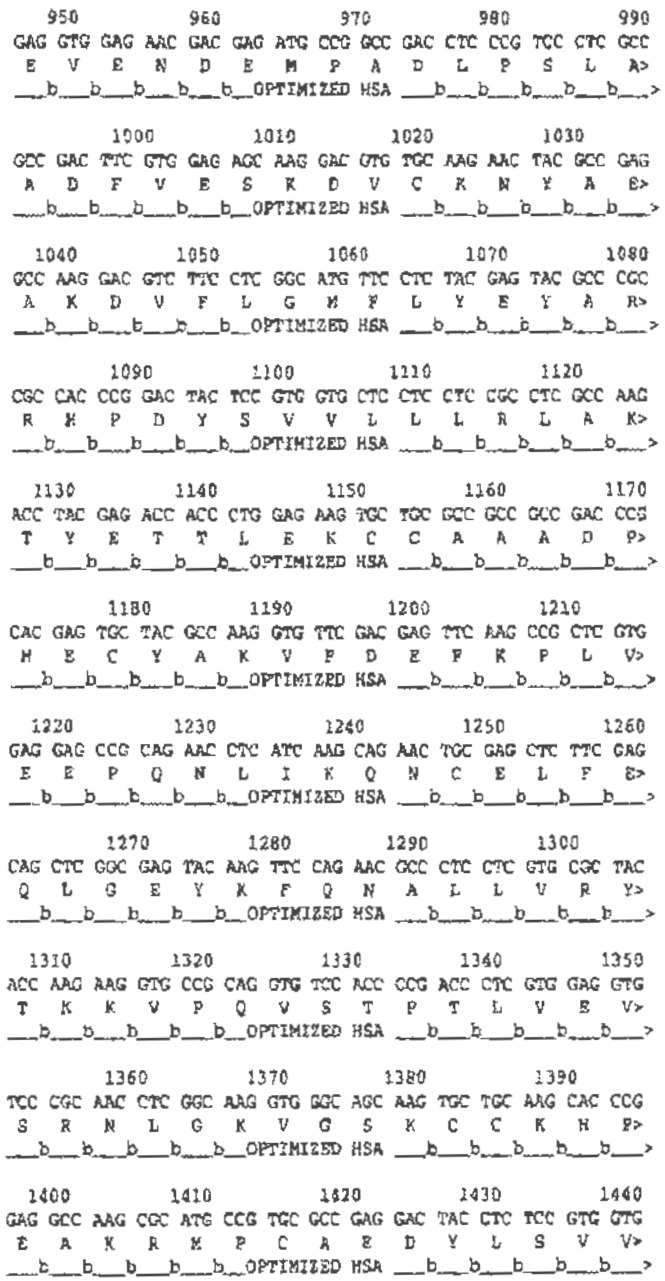

950 960 970 980 990
GAG GTG GAG AAC GAC GAG ATG CCG GCC GAC CTC CCG TCC CTC GCC
E V E N D E M P A D L P S L A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1000 1010 1020 1030
GCC GAC TTC GTG GAG AGC AAG GAC GTG TGC AAG AAC TAC GCC GAG
A D F V E S K D V C K N Y A E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1040 1050 1060 1070 1080
GCC AAG GAC GTC TTC CTC GGC ATG TTC CTC TAC GAG TAC GCC CGC
A K D V F L G M F L Y E Y A R>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1090 1100 1110 1120
CGC CAC CCG GAC TAC TCC GTG GTG CTC CTC CTC CGC CTC GCC AAG
R H P D Y S V V L L L R L A K>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1130 1140 1150 1160 1170
ACC TAC GAG ACC ACC CTG GAG AAG TGC TGC GCC GCC GCC GAC CCG
T Y E T T L E K C C A A A D P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1180 1190 1200 1210
CAC GAG TGC TAC GCC AAG GTG TTC GAC GAG TTC AAG CCG CTC GTG
H E C Y A K V F D E F K P L V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1220 1230 1240 1250 1260
GAG GAG CCG CAG AAC CTC ATC AAG CAG AAC TGC GAG CTC TTC GAG
E E P Q N L I K Q N C E L F E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1270 1280 1290 1300
CAG CTC GGC GAG TAC AAG TTC CAG AAC GCC CTC CTC GTG CGC TAC
Q L G E Y K F Q N A L L V R Y>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1310 1320 1330 1340 1350
ACC AAG AAG GTG CCG CAG GTG TCC ACC CCG ACC CTC GTG GAG GTG
T K K V P Q V S T P T L V E V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1360 1370 1380 1390
TCC CGC AAC CTC GGC AAG GTG GGC AGC AAG TGC TGC AAG CAC CCG
S R N L G K V G S K C C K H P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1400 1410 1420 1430 1440
GAG GCC AAG CGC ATG CCG TGC GCC GAG GAC TAC CTC TCC GTG GTG
E A K R M P C A E D Y L S V V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

FIG 5D

```
         1450         1460         1470         1480
CTC AAC CAG CTC TGC GTG CTC CAC GAG AAG ACC CCG GTG AGC GAC
 L   N   Q   L   C   V   L   H   E.  K   T   P   V   S   D>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1490         1500         1510         1520         1530
CGC GTG ACC AAG TGC TGC ACC GAG AGC CTC GTG AAC CGC CGC CCG
 R   V   T   K   C   C   T   E   S   L   V   N   R   R   P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1540         1550         1560         1570
TGC TTC TCC GCC CTG GAG GTC GAC GAG ACC TAC GTC CCG AAG GAG
 C   F   S   A   L   E   V   D   E   T   Y   V   P   K   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1580         1590         1600         1610         1620
TTC AAC GCC GAG ACC TTC ACC TTC CAC GCC GAC ATC TGC ACC CTC
 F   N   A   E   T   F   T   F   H   A   D   I   C   T   L>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1630         1640         1650         1660
TCC GAG AAG GAG CGC CAG ATC AAG AAG CAG ACC GCC CTC GTC GAG
 S   E   K   E   R   Q   I   K   K   Q   T   A   L   V   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1670         1680         1690         1700         1710
CTC GTG AAG CAC AAG CCG AAG GCC ACC AAG GAG CAG CTC AAG GCC
 L   V   K   H   K   P   K   A   T   K   E   Q   L   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1720         1730         1740         1750
GTG ATG GAC GAC TTC GCC GCC TTC GTG GAG AAG TGC TGC AAG GCC
 V   M   D   D   F   A   A   F   V   E   K   C   C   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1760         1770         1780         1790         1800
GAC GAC AAG GAG ACC TGC TTC GCC GAG GAG GGC AAG AAG CTC GTG
 D   D   K   E   T   C   F   A   E   E   G   K   K   L   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1810         1820         1830
GCC GCC AGC CAG GCC GCC CTC GGC CTC TGA
 A   A   S   Q   A   A   L   G   L   *>
___b___b____OPTIMIZED HSA _b___b___b___>
```

FIG. 6

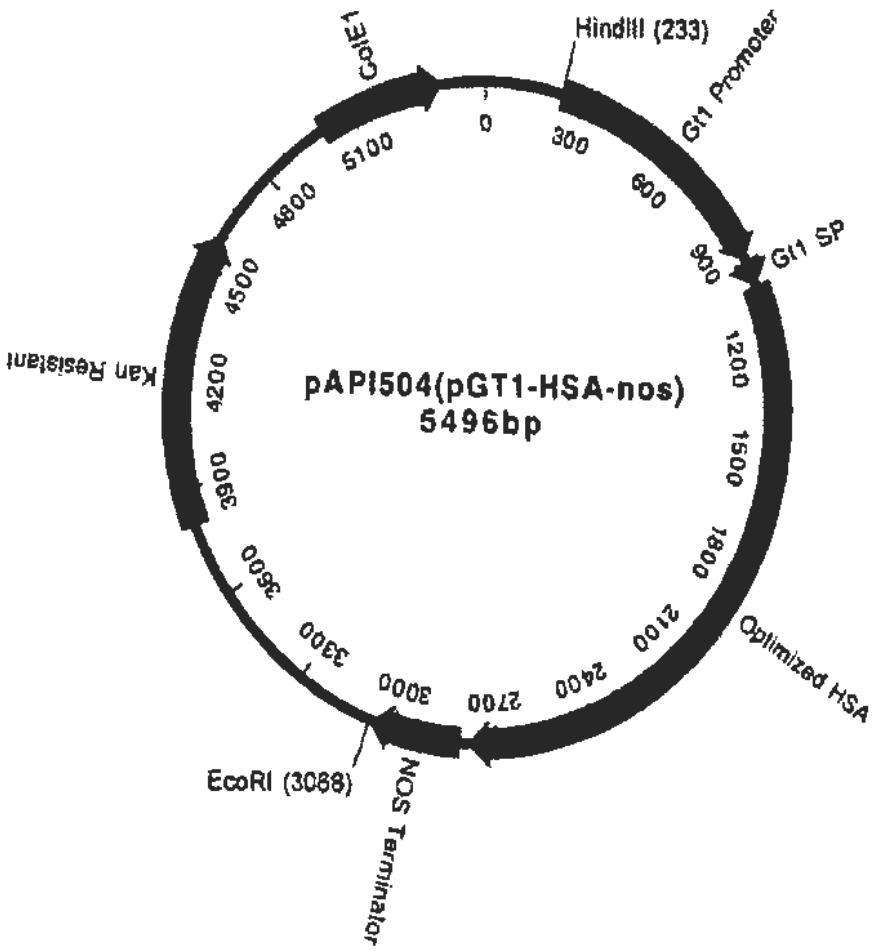

ColE1
HindIII (233)
Gt1 Promoter
Gt1 SP
Optimized HSA
NOS Terminator
EcoRI (3088)
Kan Resistant
pAPI504(pGT1-HSA-nos)
5496bp
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
3600
3900
4200
4500
4800
5100

FIG. 7

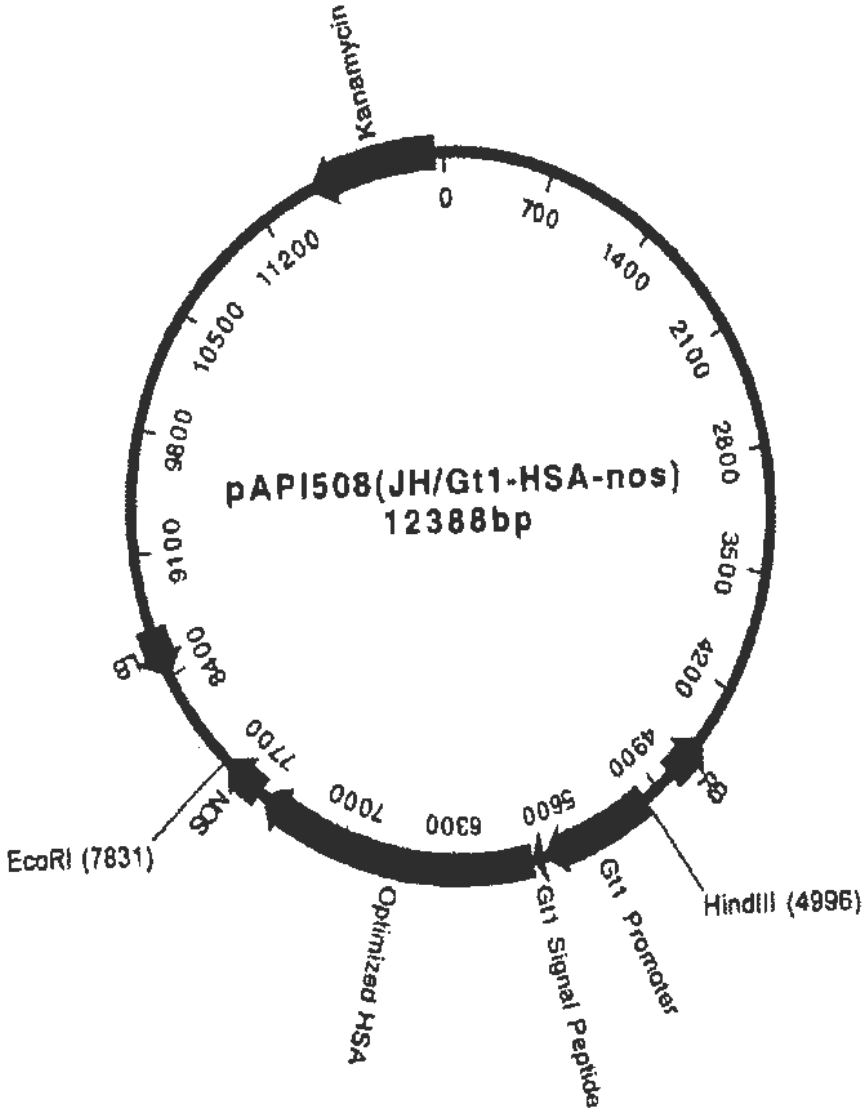

pAPI508(JH/Gt1-HSA-nos)
12388bp
Kanamycin
0
700
1400
2100
2800
3500
4200
4900
5600
6300
7000
7700
8400
9100
9800
10500
11200
pB
Gt1 Promoter
Gt1 Signal Peptide
Optimized HSA
NOS
pA
HindIII (4996)
EcoRI (7831)

FIG 8A

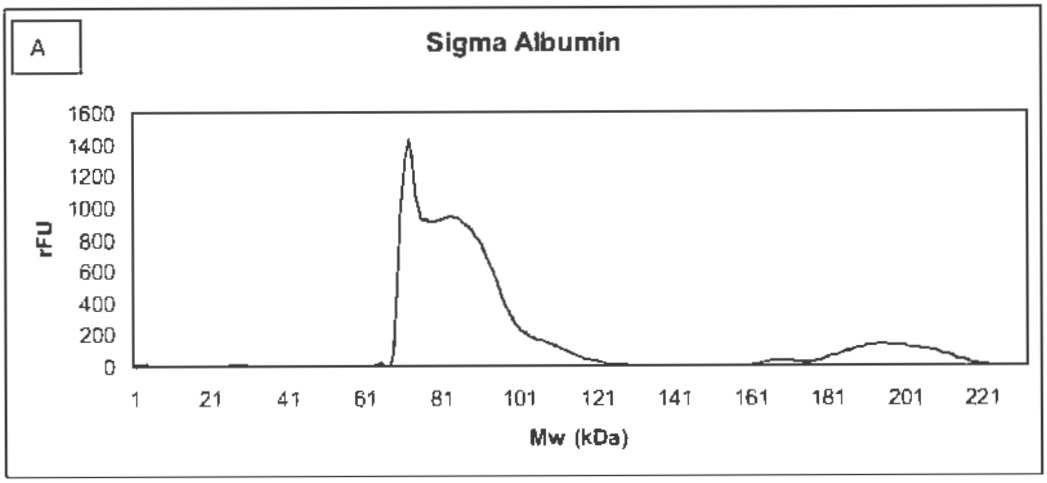


FIG 8B

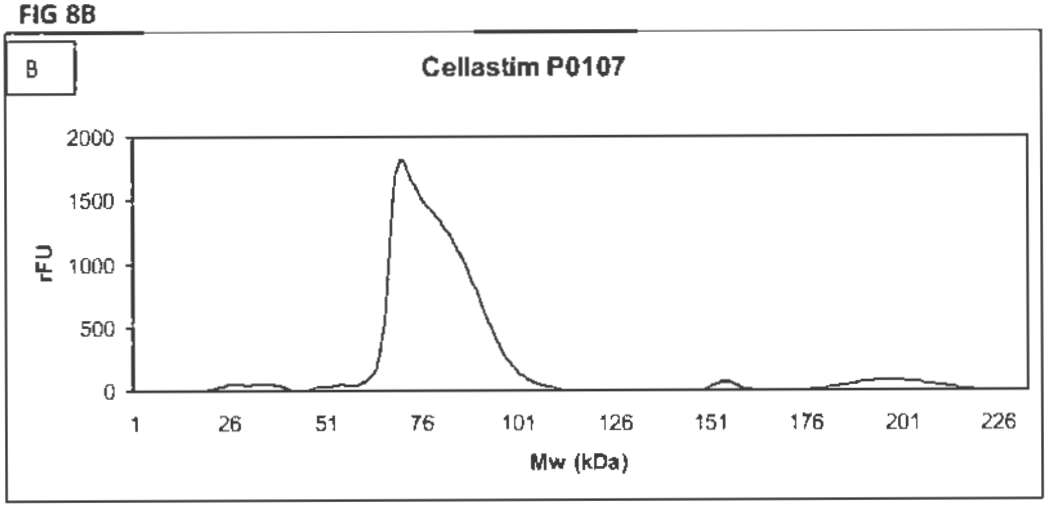


FIG 8C

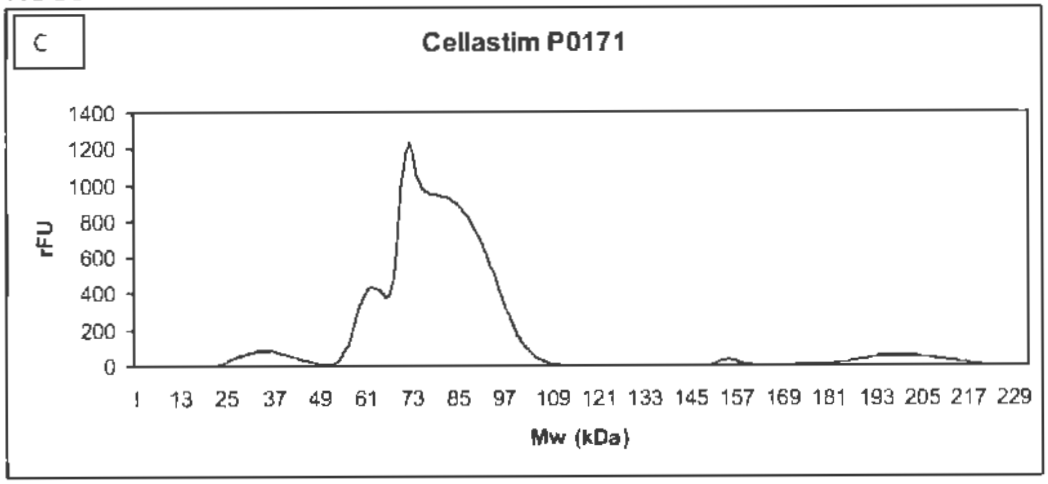


FIG 8D

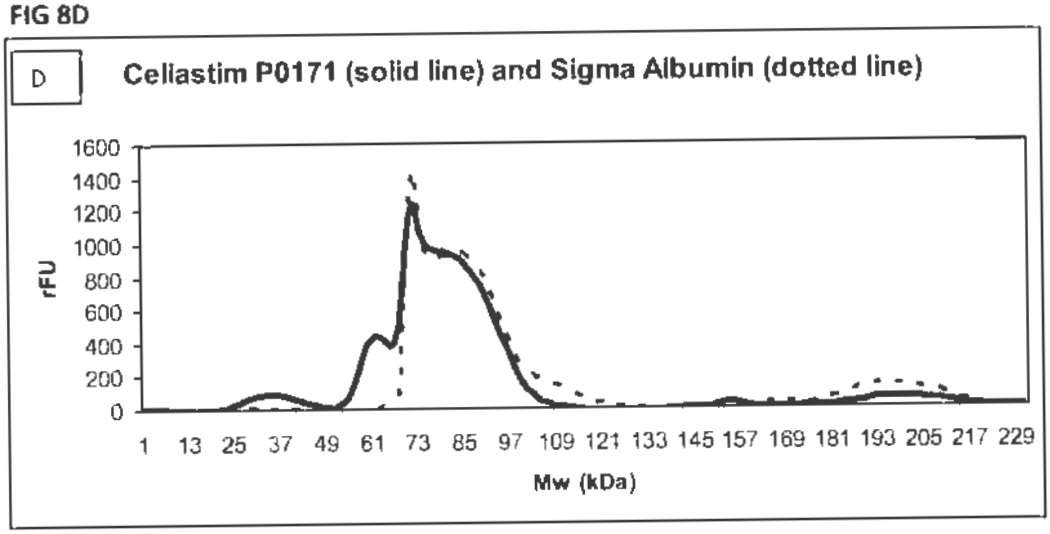

D
Celiastim P0171 (solid line) and Sigma Albumin (dotted line)
rFU
1600
1400
1200
1000
800
600
400
200
0
1 13 25 37 49 61 73 85 97 109 121 133 145 157 169 181 193 205 217 229
Mw (kDa)

FIG 8E

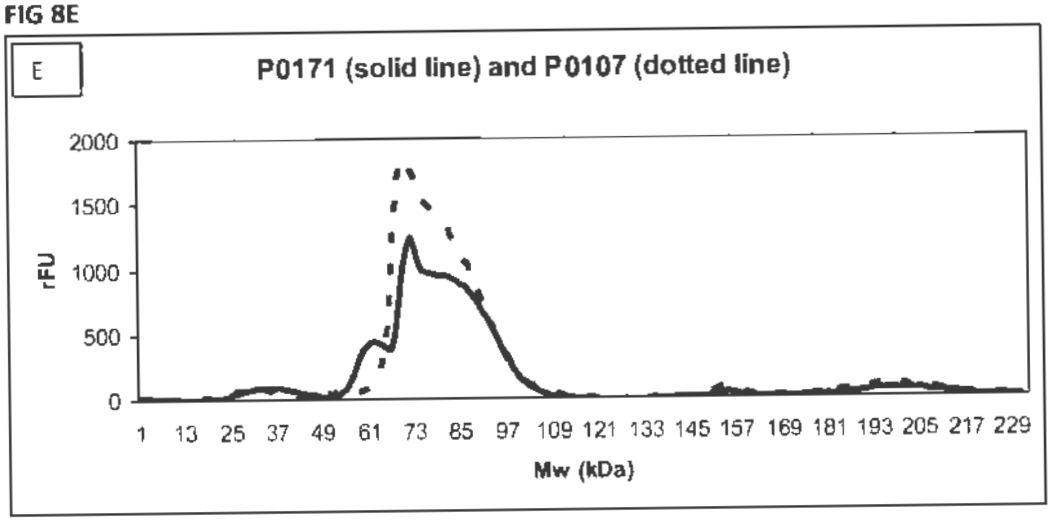

E
P0171 (solid line) and P0107 (dotted line)
rFU
2000
1500
1000
500
0
1 13 25 37 49 61 73 85 97 109 121 133 145 157 169 181 193 205 217 229
Mw (kDa)

FIG. 9A

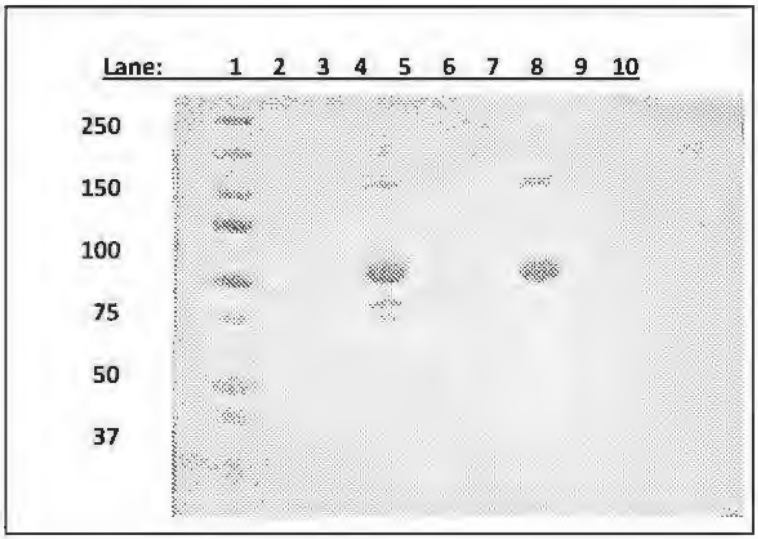

Lane: 1 2 3 4 5 6 7 8 9 10
250
150
100
75
50
37

FIG. 9B

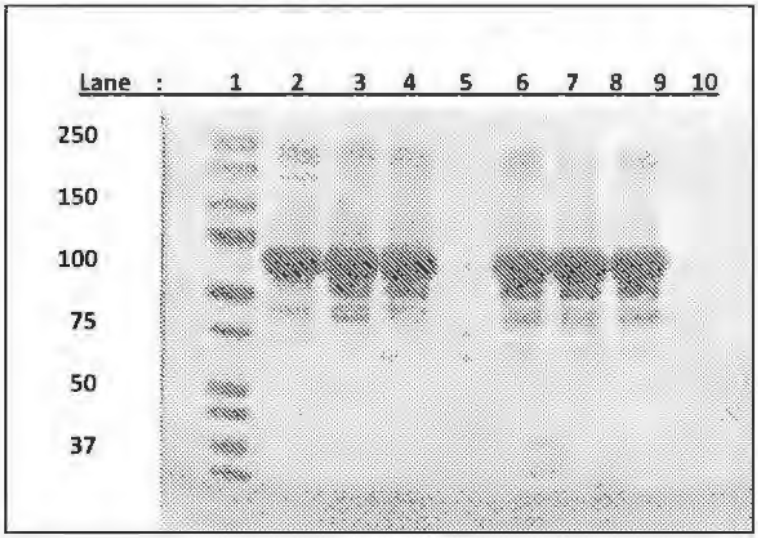

Lane : 1 2 3 4 5 6 7 8 9 10
250
150
100
75
50
37

FIG 10A

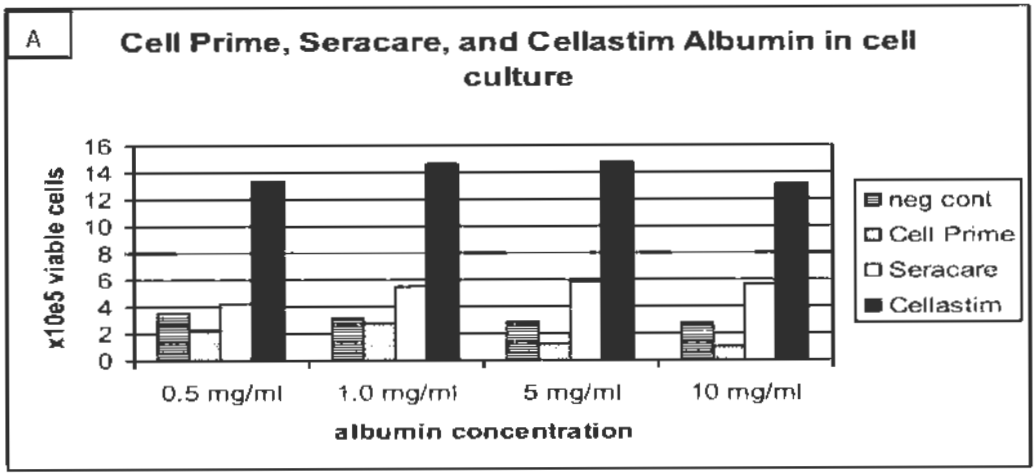


FIG 10B

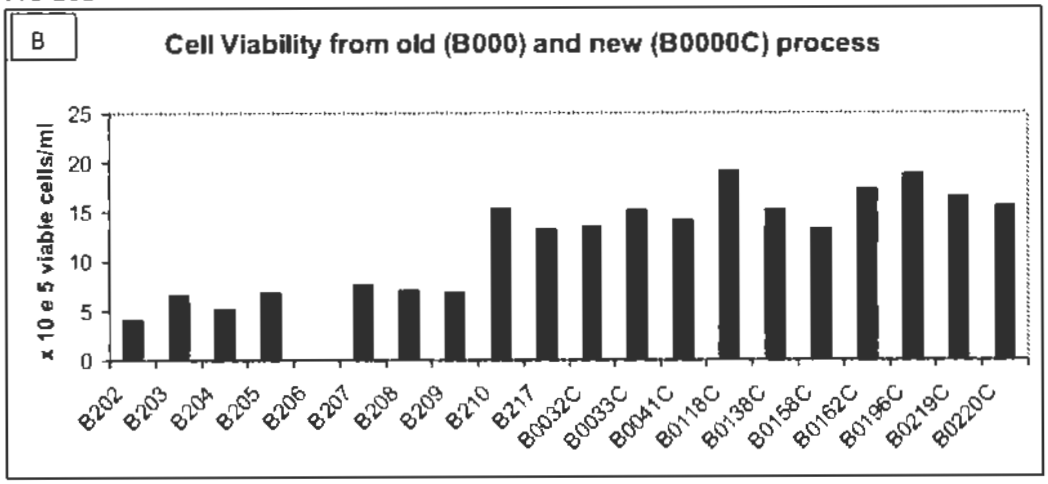


FIG 10C

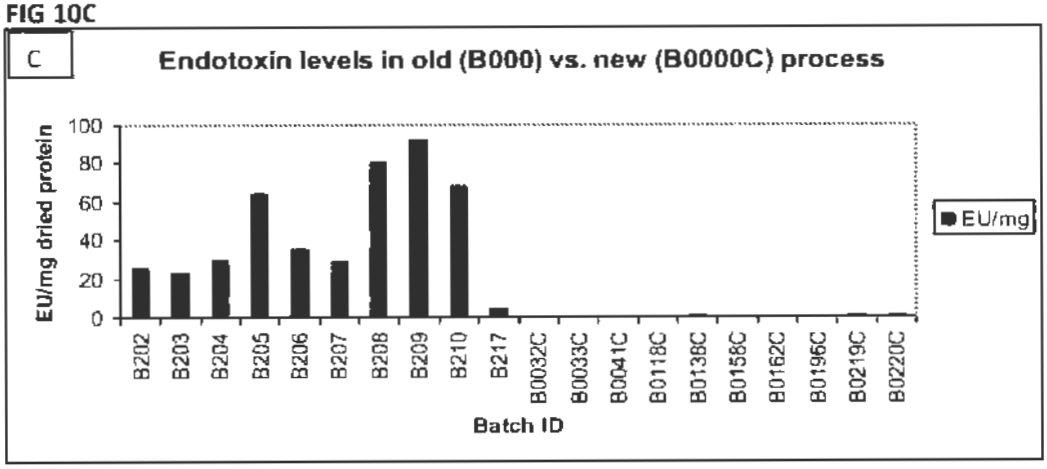


FIG 11

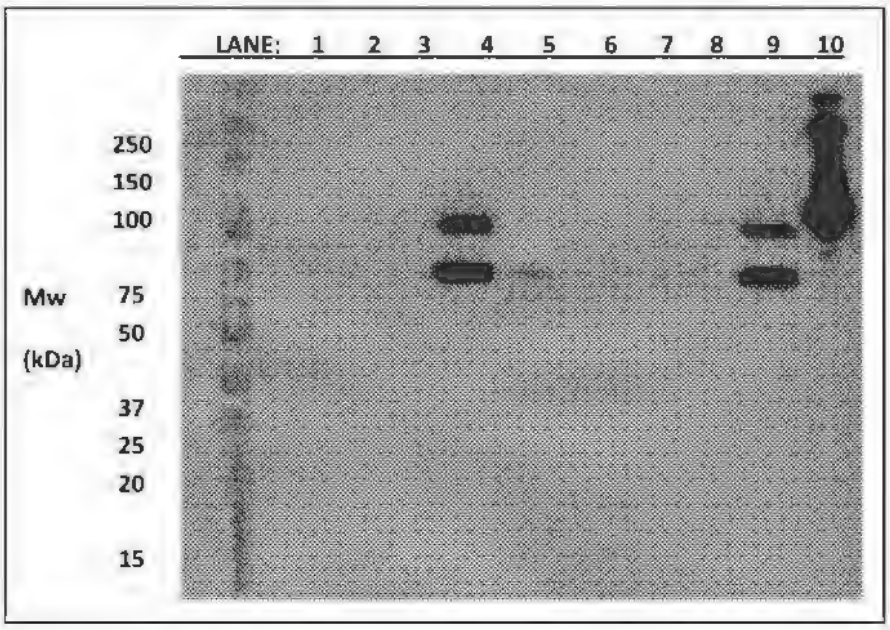


FIG. 12

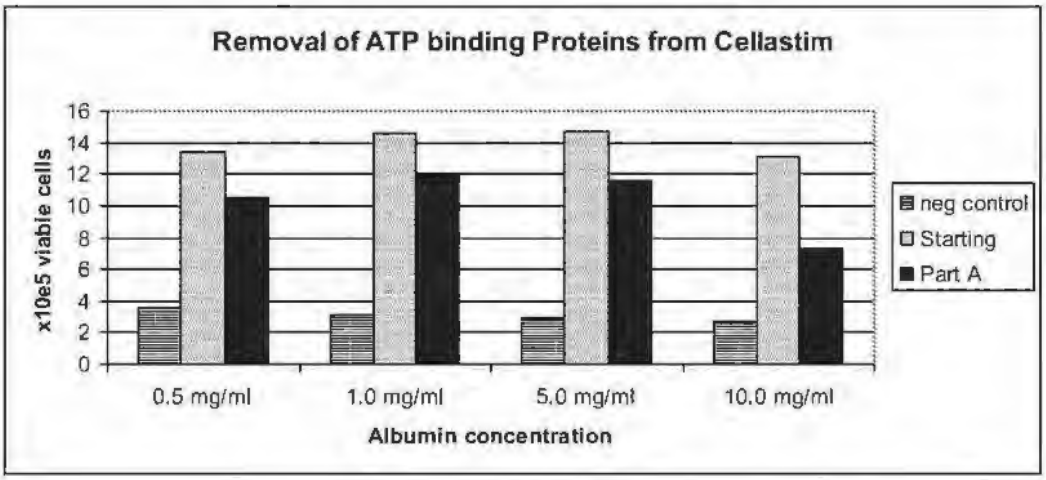
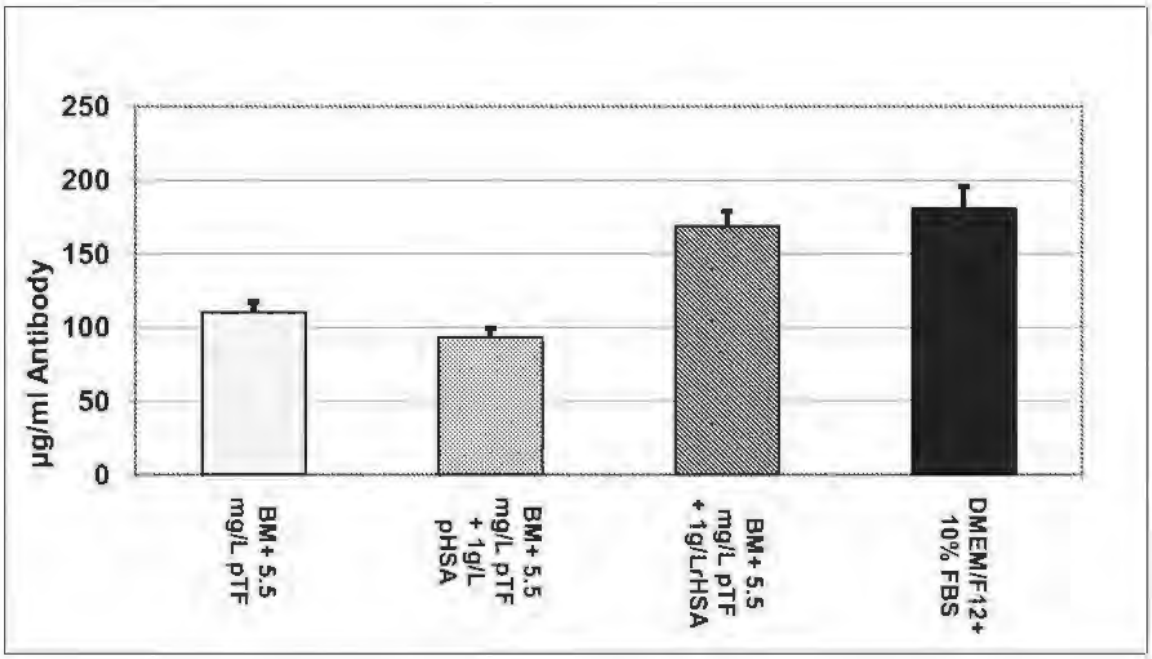

Removal of ATP binding Proteins from Cellastim
x10e5 viable cells
16
14
12
10
8
6
4
2
0
0.5 mg/ml
1.0 mg/ml
5.0 mg/ml
10.0 mg/ml
Albumin concentration
neg control
Starting
Part A

FIG. 13


250
200
150
100
50
0
µg/ml Antibody
BM+ 5.5 mg/L pTF
BM+ 5.5 mg/L pTF + 1g/L pHSA
BM+ 5.5 mg/L pTF + 1g/LrHSA
DMEM/F12+ 10% FBS

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Document code: WFEE

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Application No: 15188478 Version No: 2.0

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<110> Ventria Bioscience
Pettit, Steven C.
Santos, Mary Ann
Huang, Ming

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<130> VBS0002-201C1-US

<140> US 15/188,478
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20 25 30

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35 40 45

Ile Ala Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val
50 55 60

Lys Leu Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp
65 70 75 80

Glu Ser Ala Glu Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp
85 90 95

Lys Leu Cys Thr Val Ala Thr Leu Arg Glu Thr Tyr Gly Glu Met Ala
100 105 110

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His Lys Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val
    130                 135                 140

Asp Val Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys
145                 150                 155                 160

Lys Tyr Leu Tyr Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro
                165                 170                 175

Glu Leu Leu Phe Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys
            180                 185                 190

Cys Gln Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu
        195                 200                 205

Leu Arg Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys
    210                 215                 220

Ala Ser Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val
225                 230                 235                 240

Ala Arg Leu Ser Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser
                245                 250                 255

Lys Leu Val Thr Asp Leu Thr Lys Val His Thr Glu Cys Cys His Gly
            260                 265                 270

Asp Leu Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile
        275                 280                 285

Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu
    290                 295                 300

Lys Pro Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp
305                 310                 315                 320

Glu Met Pro Ala Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser
                325                 330                 335
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Lys Asp Val Cys Lys Asn Tyr Ala Glu Ala Lys Asp Val Phe Leu Gly
            340                 345                 350

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Val Gln Gln Leu Leu Gln Asp Phe Phe Asn Gly Lys Glu Leu Cys Lys
        355                 360                 365

Ser Ile Asn Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala
    370                 375                 380

Ala Ile Leu Ser Gly Glu Gly Asn Gln Arg Val Gln Asp Leu Leu Leu
385                 390                 395                 400

Leu Asp Val Thr Pro Leu Ser Leu Gly Leu Glu Thr Ala Gly Gly Val
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Met Thr Val Leu Ile Pro Arg Asn Thr Thr Ile Pro Thr Lys Lys Glu
            420                 425                 430

Gln Val Phe Ser Thr Tyr Ser Asp Asn Gln Pro Gly Val Leu Ile Gln
        435                 440                 445

Val Tyr Glu Gly Glu Arg Thr Arg Thr Lys Asp Asn Asn Leu Leu Gly
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Lys Phe Glu Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln
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Arg Ile Thr Pro Ser Trp Val Ala Phe Thr Asp Ser Glu Arg Leu Ile
65                  70                  75                  80

Gly Glu Ala Ala Lys Asn Gln Ala Ala Val Asn Pro Glu Arg Thr Ile
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Phe Asp Val Lys Arg Asp Ile Gly Arg Lys Phe Glu Glu Lys Glu Val
            100                 105                 110

Gln Arg Asp Met Lys Leu Val Pro Tyr Lys Ile Val Asn Lys Ile Gly
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Lys Pro Tyr Ile Gln Val Lys Ile Lys Asp Gly Glu Asn Lys Val Phe
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Ser Pro Glu Glu Val Ser Ala Met Ile Leu Gly Lys Met Lys Glu Thr
145                 150                 155                 160

Ala Glu Ala Tyr Leu Gly Lys Lys Ile Asn Asp Ala Val Val Thr Val
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Pro Ala Tyr Phe Asn Asp Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly
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Val Ile Ala Gly Leu Asn Val Ala Arg Ile Ile Asn Glu Pro Thr Ala
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Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys Gly Gly Glu Lys Asn Ile
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Leu Val Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser Ile Leu Thr
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            260                 265                 270

Leu Ile Lys Lys Lys Tyr Ser Lys Asp Ile Ser Lys Asp Asn Arg Ala
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Leu Gly Lys Leu Arg Arg Glu Ala Glu Arg Ala Lys Arg Ala Leu Ser
    290                 295                 300

Asn Gln His Gln Val Arg Val Glu Ile Glu Ser Leu Phe Asp Gly Thr
305                 310                 315                 320

Asp Phe Ser Glu Pro Leu Thr Arg Ala Arg Phe Glu Glu Leu Asn Asn
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Ala Gly Leu Glu Lys Ser Gln Ile His Glu Ile Val Leu Val Gly Gly
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Ser Thr Arg Ile Pro Lys Val Gln Gln Leu Leu Arg Asp Tyr Phe Glu
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Gly Lys Glu Pro Asn Lys Gly Val Asn Pro Asp Glu Ala Val Ala Tyr
385                 390                 395                 400

Gly Ala Ala Val Gln Gly Ser Ile Leu Ser Gly Glu Gly Gly Asp Glu
                405                 410                 415

Thr Lys Asp Ile Leu Leu Leu Asp Val Ala Pro Leu Thr Leu Gly Ile
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Glu Thr Val Gly Gly Val Met Thr Lys Leu Ile Pro Arg Asn Thr Val
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Ile Pro Thr Lys Lys Ser Gln Val Phe Thr Thr Tyr Gln Asp Gln Gln
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Asp Cys Arg Leu Leu Gly Lys Phe Asp Leu Ser Gly Ile Pro Ala Ala
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Pro Arg Gly Thr Pro Gln Ile Glu Val Thr Phe Glu Val Asp Ala Asn
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Petit	VBS0002-201C1-US	8304

72659 7590 08/03/2017

GLOBAL PATENT GROUP - VBS
17014 NEW COLLEGE AVENUE
SUITE 201
WILDWOOD, MO 63040

EXAMINER	

ART UNIT	PAPER NUMBER
1653	

NOTIFICATION DATE	DELIVERY MODE
08/03/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

admin@GLOBALPATENTGROUP.COM
lwilson@globalpatentgroup.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P O Box 1450
Alexandria VA 22313-1450
www.uspto.gov

In re Application of :
Steven Clyde Petitit, et al. :
Application No. 15/188,478 : ON PETITION
Filed: June 21, 2016 :
Attorney Docket No.: VBS0002-201C1-US :

This is a decision on the petition to revive the above-identified application, filed March 16, 2017, under the unintentional provisions of 37 CFR 1.137(a).

The petition is not signed by a registered practitioner of record; however, in accordance with 37 CFR 1.34(a), the signature of Dennis A. Bennett appearing on the petition shall constitute a representation to the United States Patent and Trademark Office that he is authorized to represent the particular party on whose behalf he acts.

The petition is **GRANTED**.

The application became abandoned for failure to reply in a timely manner to the Notice to File Missing Parts of Nonprovisional Application (Notice), mailed July 1, 2016. The Notice set a period for reply of two (2) months and required a substitute computer readable form copy of the sequence listing, replacement drawings, the statutory basic filing fee, search fee and examination fee, additional claim fees and surcharge under 37 CFR 1.16(f). A Notice of Abandonment was mailed on March 2, 2017. On March 16, 2017, the present petition was filed, including, inter alia, a five (5) month petition for extension of time.

Extensions of time under 37 CFR 1.136 are available only if asked for prior to or within the response period set by statute. In no case, however, may an applicant respond later than the maximum time period set by statute. The extension of time submitted March 16, 2017, was filed when the provisions of 37 CFR 1.136 could no longer be used. Therefore, this application became abandoned when the unextended time for response had expired. Since no extension of time fees are due on a petition for revival, the $1500 extension fee submitted after abandonment is subject to refund.

Any request for refund must include a copy of this decision and be mailed to Mail Stop 16, Director of the U.S. Patent and Trademark Office, P. O. Box 1450, Alexandria, VA 22313-1450 or faxed to the Customer Service Help Desk at (571) 273-6500.

The petition satisfies the requirements of 37 CFR 1.137(a) in that petitioner has supplied (1) the reply in the form of the replacement drawings, a substitute sequence listing, the requisite filing fee, search fee, examination fee and surcharge, (2) the petition fee of $850, and (3) an adequate statement of unintentional delay.

The application is being referred to the Office of Patent Application Processing (OPAP) for further pre-examination processing.

Telephone inquiries related to this decision should be directed to the undersigned at (571) 272-3204. Telephone inquiries related to processing at OPAP should be directed to their hotline at (571) 272-4000.

/SDB/
Sherry D. Brinkley
Lead Paralegal Specialist
Office of Petitions

PTO/AIA/80 (07-12)
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U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

☒ Practitioners associated with Customer Number: 23589

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named then a customer number must be used):

Name	Registration Number

Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

☒ The address associated with Customer Number: 23589

OR

☐	Firm or Individual Name		
	Address		
	City	State	Zip
	Country		
	Telephone	Email	

Assignee Name and Address: Ventria Bioscience Inc.
2718 Industrial Drive
Junction City, KS 66441

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	Scott E. Deeter	Date	18th day of July, 2017
Name	Scott E. Deeter	Telephone	970-420-9598
Title	President and CEO		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	30348322
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	72659
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	VBS0002-201C1-US
Receipt Date:	13-SEP-2017
Filing Date:	21-JUN-2016
Time Stamp:	12:07:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Assignee showing of ownership per 37 CFR 3.73	PTO_373_Statement_13Sept17_50091-CNT_957158.PDF	104839 [illegible]	no	3

Warnings:

Information:					
2	Power of Attorney	PTO_POA_13Sept17_50091-CNT_957149.PDF	96278	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	201117		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/96 (08-12)
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STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Ventria Bioscience Inc.

Application No./Patent No.: 15/188,478 Filed/Issue Date: June 21, 2016

Titled: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

Ventria Bioscience Inc., a corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☑ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☑ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 040726, Frame 0310, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

   1. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

   2. From: _____ To: _____

      The document was recorded in the United States Patent and Trademark Office at

      Reel _____, Frame _____, or for which a copy thereof is attached.

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Crissa A. Cook/	2017-09-13
Signature	Date
Crissa A. Cook	64,054
Printed or Typed Name	Title or Registration Number

[Page 2 of 2]

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
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3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	VBS0002-201C1-US

CONFIRMATION NO. 8304

POA ACCEPTANCE LETTER

23589
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

OC000000094517162

Date Mailed: 10/04/2017

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 09/13/2017.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/vostuart/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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[illegible]

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	VBS0002-201CT-US

CONFIRMATION NO. 8304

POWER OF ATTORNEY NOTICE



72659
GLOBAL PATENT GROUP - VBS
17014 NEW COLLEGE AVENUE
SUITE 201
WILDWOOD, MO 63040

Date Mailed: 10/04/2017

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 09/13/2017.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/vostuart/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Petti	50091-CNT1	8304

23589 7590 05/10/2018

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER
BOLLAND, PAUL I

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
05/10/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

uspatents@hoveywilliams.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P O Box 1450
Alexandria VA 22313-1450
www.uspto.gov

In re Application of
Steven Clyde Petitit, et al.
Application No. 15/188,478
Filed: June 21, 2016
Attorney Docket No.: VBS0002-201C1-US

ON PETITION

The above-identified application was recently returned to the Office of Petitions for reconsideration of the decision mailed August 3, 2017. Accordingly, this is a corrected decision on the petition to revive the above-identified application, filed March 16, 2017, under the unintentional provisions of 37 CFR 1.137(a).

The petition is **DISMISSED**.

Any request for reconsideration of this decision must be submitted within TWO (2) MONTHS from the mail date of this decision. Extensions of time under 37 CFR 1.136(a) are permitted. The reconsideration request should include a cover letter entitled "Renewed Petition under 37 CFR 1.137(b)." This is **not** a final agency action within the meaning of 5 U.S.C. § 704.

The application became abandoned for failure to reply in a timely manner to the Notice to File Missing Parts of Nonprovisional Application (Notice), mailed July 1, 2016. The Notice set a period for reply of two (2) months and required a substitute computer readable form copy of the sequence listing, replacement drawings, a substitute specification, the statutory basic filing fee, search fee and examination fee, additional claim fees and surcharge under 37 CFR 1.16(f). A Notice of Abandonment was mailed on March 2, 2017. On March 16, 2017, the present petition was filed.

A grantable petition under 37 CFR 1.137(a) must be accompanied by: (1) the required reply, unless previously filed; (2) the petition fee as set forth in 37 CFR 1.17(m); (3) a statement that the entire delay in filing the required reply from the due date for the reply until the filing of a grantable petition pursuant to 37 CFR 1.137(a) was unintentional. Where there is a question as to whether either the abandonment or the delay in filing a petition under 37 CFR 1.137 was unintentional, the Director may require additional information. See MPEP 711.03(c)(II)(C) and (D).

The petition does not comply with item (1). In this regard, a complete response to the Notice of July 1, 2016 was not submitted, in that a substitute specification in compliance with 37 CFR 1.52, 1.121 (b)(3), and 1.125, including a statement that the substitute specification contains no new matter, was not filed.

In view of the above, the decision mailed August 3, 2017 is hereby vacated.

Further correspondence with respect to this matter should be delivered through one of the following mediums:

By mail:	Mail Stop PETITIONS Commissioner for Patents Post Office Box 1450 Alexandria, VA 22313-1450
By hand:	Customer Service Window Mail Stop Petitions Randolph Building 401 Dulany Street Alexandria, VA 22314
By fax:	(571) 273-8300 ATTN: Office of Petitions
By internet:	EFS-Web[1]

Telephone inquiries related to this decision should be directed to the undersigned at (571) 272-3204.

/SDB/
Sherry D. Brinkley
Lead Paralegal Specialist
Office of Petitions

[1] www.uspto.gov/ebc/efs_help.html (for help using EFS-Web call the Patent Electronic Business Center at (866) 217-9197)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Art Unit: 1656
Filing Date: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. Holland

Mail Stop PETITIONS
Attn: Sherry D. Brinkley
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

RENEWED PETITION UNDER 37 CFR 1.137(b)

The present Renewed Petition is in reply to the Decision mailed in the above-referenced application on May 10, 2018 dismissing the Petition for Revival of an unintentionally abandoned application. The Decision indicated that the prior petition and response dated March 16, 2017 were incomplete and did not include a substitute specification in compliance with the rules and statement regarding new matter. The present Renewed Petition is being submitted with a substitute specification in both clean and marked-up copies, along with the requisite statement. **It is reconfirmed that the entire delay in filing the required reply from the due date for the required reply until the filing of a grantable petition under 37 C.F.R. 1.137(a) was unintentional.**

The undersigned has recently assumed responsibility for this application from the prior law firm. A notice *granting* the original petition for revival was previously mailed on August 3, 2017; and accordingly, Applicant believed the current application to be presently pending. Upon reconsideration, the Office determined that the prior notice was in error. Based upon the prior correspondence and a telephone call with Ms. Brinkley on July 6, 2018, the remaining issue in the present application relates to the description of the Figures in the specification, which needed to be amended to match the updated name in the Replacement Drawings.

Applicant notes that a Preliminary Amendment was included with the petition filed on March 16, 2017. The Preliminary Amendment amended the claims and updated the cross-reference to related applications, as well as amending the description of the Figures. **Applicant**

requests entrance of those claim amendments for fee calculation purposes and examination. The specification amendments have been corrected and are reflected in the substitute specification submitted herewith.

It is believed that the other matters noted by the prior Missing Parts have been satisfied, and the application can now proceed to examination. However, should anything further be required, the Office is encouraged to contact the undersigned.

Any fee which is due in connection with this response should be applied against Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Cook/

By _____

Crissa A. Cook, Reg. No. 64,054
HOVEY WILLIAMS LLP
10801 Mastin Boulevard, Suite 1000
Overland Park, KS 66210
913-647-9050
ATTORNEYS FOR APPLICANT(S)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Art Unit: 1656
Filing Date: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. Holland

Mail Stop PETITIONS
Attn: Sherry D. Brinkley
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

PRELIMINARY AMENDMENT AND STATEMENT

In response to the Decision mailed in the above-referenced application on May 10, 2018 dismissing the Petition for Revival of an unintentionally abandoned application, amendment and reconsideration of this application is requested.

Amendments to the Specification begin on page 2.

Remarks begin on page 3.

Amendments to the Specification:

Please replace the originally-filed specification with the substitute specification, without claims, submitted herewith.

Remarks.

A substitute specification, without claims, is submitted herewith in both clean and marked up copies pursuant to the applicable rules.

STATEMENT: The amendments to the specification contain no new matter.

In the substitute specification, the references to the "Figures" has been corrected to match the naming in the Replacement Drawings that were previously submitted. Accordingly, each instance of "Figure" has been replaced with "FIG." The Brief Description of the Figures has also been corrected to refer specifically to each figure in the drawings.

The cross-reference to related applications section has been updated to refer to the parent application.

In addition to these defects, the specification has also been corrected to remove a webpage code "http" from page 2. A period has been added at the end of the sentence before each table on pages 54, 55, and 56. Formatting has also been removed on page 77. The shading has also been removed from the tables throughout the specification. Further, several portions of the original specification contained underlined text (e.g., headings). To avoid confusion, this underlining has been removed. As such, only added content is shown as being underlined in the marked-up copy of the specification. There is no underlined content in the clean copy of the specification.

Again, the changes to the specification include no new matter.

Finally, as noted in the renewed petition submitted herewith, a Preliminary Amendment was filed on March 16, 2017, containing an **amended set of claims**. Applicants respectfully request **entrance of these amendments for fee calculation and examination purposes**.

It is believed that the present application should now be complete and ready for examination.

Any fee which is due in connection with this response should be applied against Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Cook/

By _____

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ATTORNEYS FOR APPLICANT(S)

Cell Culture Media Containing Combinations of Proteins

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Serial No. 12/798,462, filed February 18, 2010, which claims the benefit of US provisional patent application No. 61,154,204, filed February 20, 2009, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

BACKGROUND OF THE INVENTION

Cell culture techniques allow animal or plant cells that are removed from tissues to grow when supplied with the appropriate nutrients and conditions. The cells are capable of dividing and can continue to grow until limited by culture variables such as nutrient depletion or toxic buildup (Butler, M. & Jenkins, H., "Nutritional aspects of growth of animal cells in culture," J of Biotechnol. (1989) 12: 97-110). Cell culture techniques have a number of applications including investigation of the normal physiology or biochemistry of cells (Balaban, B. & Urman, B., "Embryo culture as a diagnostic tool," Reprod. Biomed. Online (2003) 7(6): 671-82), testing the effect of various chemical compounds or drugs on specific cell types (Farkas, D. & Tannenbaum, S.R., "In vitro methods to study chemically-induced hepatotoxicity: a literature review," Curr. Drug Metab. (2005) 6(2): 111-25), studying the sequential or parallel combination of various cell types to generate artificial tissues (Wang et al., "Cartilage tissue engineering with silk scaffolds and human articular chondrocytes," Biomaterials (2006)), and synthesizing valuable biologics from large scale cell cultures (Zeilinger et al., "Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions," Altern. Lab Anim. (2002) 30(5): 525-38). Cell culture techniques have also been used for in vitro fertilization (Blake et al., "Protein supplementation of human IVF culture media," J Assist Reprod. Genet. (2002) 19(3): 137-43; Bungum et al., "Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study," Reprod. Biomed. Online (2002) 4(3): 233-6), stem cell research (Conley et al., "Derivation, propagation and differentiation of human embryonic stem cells," Int. J Biochem Cell Biol. (2004) 36(4): 555-67), vaccine production (Chuang et al., "Pharmaceutical strategies utilizing recombinant human albumin," Pharm. Res. (2002) 19(5): 569-77; GlaxoSmithKline, HAVRIX® (Hepatitis A Vaccine, Inactivated) - Prescribing Information

(2005), available at us.gsk.com/products/assets/us_havrix.pdf; Innis et al., "Protection against hepatitis A by an inactivated vaccine," JAMA (1994) 271(17): 1328-34; Merck, PROQUAD® - Measles, Mumps, Rubella, and Varicella (Oka/Merck) Virus Vaccine Live - Prescribing Information (2005), available at www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf; Litwin, J., "The growth of Vero cells in suspension as cell-aggregates in serum-free media," Cytotechnology (1992) 10(2): 169-74), tissue engineering including artificial skin (Atala, A., "Future perspectives in reconstructive surgery using tissue engineering," Urol. Clin. North Am. (1999) 26(1): 157-65, ix-x; Sher, et al., "Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation," J. Biol. Chem. (2006) 281(8): 5178-87) and organs (Neronov et al., "Integrity of endothelium in cryopreserved human cornea," Cryo Letters (2005) 26(2): 131-6; Han, et al., "Interleukin-1 alpha-induced proteolytic activation of metalloproteinase-9 by human skin," Surgery (2005) 138(5): 932-9) and gene and cell therapy (Chadd, H.E. & Chamow, S.M., "Therapeutic antibody expression technology," Curr. Opin. Biotechnol. (2001) 12(2): 188-94).

Biologics encompass a broad range of cell products, and include specific proteins or viruses that require animal cells for propagation. For example, therapeutic proteins such as monoclonal antibodies can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures (Dewar et al., "Industrial implementation of in vitro production of monoclonal antibodies, Ilar J. (2005) 46(3): 307-13). The number of such commercially valuable biologics has increased rapidly over the last decade and has led to the present widespread interest in mammalian cell culture technology (Mizrahi, A., "Biologicals produced from animal cells in culture – an overview," Biotechnol. Adv. (1988) 6(2): 207-20).

The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The need for cell culture, especially at large scale, became apparent with the need for viral vaccines. Major epidemics of polio in the 1940s and 1950s promoted efforts to develop an effective vaccine. In 1949, it was shown that poliovirus could be grown in cultures of human cells, which led to considerable interest in the development of large quantities of the polio vaccine using cell culture (Ligon, B.L., "Thomas Huckle Weller MD: Nobel Laureate and research pioneer in poliomyelitis, Varicella-zoster virus, cytomegalovirus, rubella, and other infectious diseases," Semin. Pediatr. Infect. Dis. (2002) 13(1): 55-63). The polio vaccine, produced from de-activated virus, became one of the first commercial products of cultured animal cells (Furesz, J., "Developments in the production and quality control of poliovirus vaccines – Historical perspectives," Biologicals (2006))

Due to the safety and ethical considerations associated with the use of animal-derived cell culture media components, efforts have been made to provide alternative sources for cell culture media and media components

Published PCT Appl. No. WO 2007/002762, which was based on U.S. Provisional Appl. No. 60/694,236, filed June 28, 2006, relates to recombinant production of components of cell culture media using plant cells, and cell culture media containing such recombinant proteins. The entire contents of this application are incorporated herein by reference.

SUMMARY OF THE INVENTION

Accordingly, it is an objective of the invention to provide cell culture media or media supplements containing combinations of protein components, which when used in cell culture, result in improved growth and/or productivity of cultured cells.

One embodiment of the invention is a cell culture media including a cell culture media base, and two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

Yet another embodiment of the invention is a cell culture media including a cell culture media base, albumin, and at least one additional protein selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the growth factors that may be provided in the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

Another embodiment of the invention is a cell culture media including a cell culture media base, lactoferrin, and at least one additional protein selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to further embodiments, a preferred combination of proteins that may be provided in the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides methods for producing supplemented cell culture media, including providing a cell culture media base; providing two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin; and combining the two or more proteins with the cell culture media base to form the supplemented cell culture media.

According to further embodiments, the proteins used in the methods for producing cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be provided in the methods of producing the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of producing the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides a method for culturing cells, including providing a cell culture media base, supplementing the cell culture media base with two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin to form a supplemented cell culture media, and introducing cells to be cultured into the supplemented cell culture media. The cells grown in the supplemented cell culture media exhibit improved growth characteristics as compared to cells grown in an unsupplemented cell culture media.

According to further embodiments, the proteins used in the methods for culturing cells are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for culturing cells are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be used in the methods for culturing cells are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, by culturing the cultured cell in the cell culture media of the present invention

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100,000, to about 1 to 1,000.

In one aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein. In aspect of this embodiment, the heat shock protein is a rice heat shock protein. In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein. In another aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469

In another aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt/wt HSP70.

In another aspect of any of the claimed supplements, the transferrin related protein is lactoferrin. In another aspect of any of the claimed supplements, the transferrin related protein is transferrin. In one aspect, of any of the claimed supplements the lactoferrin is human. In one aspect, of any of the claimed supplements the transferrin is human. In one aspect, of any of the claimed supplements the lactoferrin is recombinant. In one aspect, of any of the claimed supplements the lactoferrin is derived from plasma. In one aspect, of any of the claimed supplements the transferrin is recombinant. In one aspect, of any of the claimed supplements the transferrin is derived from plasma. In one aspect, of any of the claimed supplements the IGF-1 is human. In one aspect, of any of the claimed supplements the IGF-1 is recombinant.

In another aspect of any of the claimed supplements, the ratio of transferrin to albumin about 1 to 50, to about 1 to 200. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 75, to about 1 to 180 In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 80, to about 1 to 120. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 1000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 2000. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 3000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 4000. in another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 5000.

In another aspect of any of the claimed supplements. the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0 5, to about 1 to 2 In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and a

transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises. a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein. wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of

albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin. and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the cell culture medium wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a

plant. ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated

bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 5000. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In one aspect of any of these methods, the supplement further comprises insulin. In one aspect of any of these methods, the supplement further comprises IGF-1. In another aspect, the supplement further comprises selenium and ethanolamine.

In another aspect of any of the claimed methods, the cells are tissue culture cells. In another aspect, the cells are CHO cells. In another aspect the cells are hybridoma cells. In another aspect the cells are vero cells. In another aspect the cells are sorted by flow cytometry. In another aspect the cells are primary cells. In one aspect, the primary cells are embryonic stem cells. In another aspect the primary cells are B-cell derived. In another aspect the cells the primary cells are T-cell derived. In another aspect the cells are B-cells or B-cell derived. In another aspect the cells are T-cells or T-cell derived. In another aspect the cells. In another aspect the cells are isolated by a micro fluidic device. In another aspect the cells are isolated by single-cell subcloning

In one aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt / wt of a heat shock protein In aspect of this embodiment, the heat shock protein is a rice heat shock protein In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein In another

aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / Osl_37938, and AAB63469.

In another aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt /wt HSP70

In another aspect of any of the claimed methods, the transferrin related protein is lactoferrin. In another aspect of any of the claimed methods the transferrin related protein is transferrin. In one aspect, of any of the claimed methods the lactoferrin is human. In one aspect, of any of the claimed methods the transferrin is human. In one aspect, of any of the claimed methods the lactoferrin is recombinant. In one aspect, of any of the claimed methods the transferrin is recombinant.

In another aspect of any of the claimed methods, the ratio of transferrin to albumin is about 1 to 50, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 75, to about 1 to 180. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 80, to about 1 to 120. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 120, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 200, to about 1 to 1000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 1000, to about 1 to 2000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 2000, to about 1 to 3000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 3000, to about 1 to 4000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 4000, to about 1 to 5000.

In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.5, to about 1 to 2. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 100 mg /L and 400 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 400 mg /L and 800 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 800 mg /L and 1000mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 1000 mg /L and 2000 mg/ L.

In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 1ug /L and 50 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 2 ug /L and 10 ug/ L In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 3 ug /L and 8 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 4 ug /L and 6 ug/ L.

In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 100 mg /L and 2000 mg/ L. In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 200 mg /L and 1000 mg/ L.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 30 % compared to the summed effect of recombinant albumin and transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 40 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 50 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 60 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 70 % compared to the summed effect of recombinant albumin or transferrin related protein alone when measured under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 80 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 90 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 100 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 150 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions

In another aspect of any of the claimed methods the improvement in cell viability is greater than 200 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

Other novel features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

BRIEF DESCRIPTION OF THE FIGURES

For a more complete understanding of the present invention, the needs satisfied thereby, and the objects, features, and advantages thereof, reference now is made to the following description taken in connection with the accompanying drawings.

FIG. 1A is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1B is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1C is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1D is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 2 is a plasmid map of the 5,817 bp plasmid, API164 (GT1-Lac), showing an expression cassette for human lactoferrin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human lactoferrin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 3 shows the results of a SDS-PAGE analysis for the expression of recombinant human lactoferrin. Total proteins from rice seed extracts were suspended in Laemli sample buffer, run on a gradient gel and stained with Coomassie blue. Lane 1 is the molecular weight marker. Lanes 3 to 6 are purified human derived lactoferrin (Sigma Chemical, USA). Lanes 8 to 13 are single seed extracts from homozygous independent transgenic rice lines and lane 14 is a seed extract from non-transformed rice variety Taipei 309.

FIG. 4 is a stable expression of recombinant human lactoferrin in transgenic rice grains from R_2 through R_{10} generations. Total soluble proteins from 1 g of brown rice flour was extracted with 40 ml of extraction buffer and clarified by centrifugation. The extract was analyzed via ELISA. Extraction was repeated three times and standard deviation is shown as an error bar.

FIG. 5A shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5B shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5C shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot)

FIG. 5D shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 6 is a plasmid map of the 5,496 bp plasmid, API504 (GT1-HSA), showing an expression cassette for human albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 7 is a plasmid map of the 12,388 bp plasmid, API508 (JH/GT1-HSA), showing an expression cassette for human serum albumin (HSA) and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 8A-FIG. 8E Shows a comparison by HPLC size exclusion chromatography of recombinant HSA produced from rice compared to other sources of albumin and methods of purification. **FIG. 8A** shows the chromatogram for a serum derived (non-recombinant HSA). **FIG. 8B** shows the chromatogram for a rice recombinant HSA (Cellastim P0107) made using the "old process" B000 for purification. **FIG. 8C** shows the chromatogram for a rice recombinant HSA (Cellastim P0171) made using the "new process" B0000C for purification. **FIG. 8D** shows an overlay of the chromatograms for the serum derived albumin (1A; dotted line) and Cellastim HSA prepared using the new process ((1C; solid line). **FIG. 8E** shows an overlay of the chromatograms for HSA prepared using the old process B000 (Cellastim P0107) (1B, dotted line) and HSA prepared using the new process B0000C (Cellastim P0171) (1C; solid line).

FIG. 9A-FIG. 9B Shows a comparison by SDS PAGE analysis of recombinant albumin produced from rice compared to other sources of HSA and methods of purification **FIG. 9A** shows a comparison of Cellastim P0171albumin and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **FIG. 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

FIG. 10A: Shows a comparison of the effects of yeast recombinant HSA (Cellprime), human plasma derived HSA, (Seracare) and plant recombinant HSA (Cellastim P0171) with respect to their ability to promote cell growth and viability. **FIG. 10B** shows a comparison of the endotoxin levels in batches of albumin produced using the old (B000) and new processes (B0000C) for recombinant albumin production. **FIG. 10C** shows a comparison of cell growth and viability of cells grown in the presence of the Cellastim HSA produced using the old (B000) and new processes (B0000C) for recombinant albumin production.

FIG. 11: Shows a western blot of two independent experiments showing that plant derived recombinant HSA comprises proteins that can be selectively concentrated by an ATP affinity column (Lanes 4 & 9) and react against an anti-HSP antibody. Lanes 3 and 7 show 10 ug of starting material; Lanes 4 and 9 show the fractions after elution and concentration from the ATP affinity column; Lanes 5 and 8 show the wash through after elution with ATP, and lane 10 is a hsp positive control. (See Example 9 for details).

FIG. 12: Shows a comparison of the cell growth and viability effect of Cellastim recombinant albumin after passing the albumin produced using the new process over an ATP affinity column to remove heat shock proteins. (See text for details)

FIG. 13: Shows a comparison of the effect of 1 g/L recombinant plant derived human serum albumin (rHSA) in combination with transferrin (pTF) compared to the same concentration of plasma derived human serum albumin (pHSA) in combination with transferrin (pTF) on the production of antibody by hybridoma cells in culture.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description. Unless otherwise indicated, all terms used herein have the meanings given below, or are generally consistent with same meaning that the terms have to those skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds. (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands, for definitions and terms of the art.

The term "**about**" or "**approximately**" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviations, per practice in the art. Alternatively, "about" with respect to the compositions can mean plus or minus a range of up to 20%, preferably up to 10%, more preferably up to 5%.

The term "**antigen-binding fragment**" refers to a polypeptide portion of an immunoglobulin or antibody that binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). Binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies.

The term "**apoptosis**" ("normal" or "programmed" cell death) refers to the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine dependent tissue atrophy. Apoptosis may also be triggered in cells grown under tissue culture conditions in response to stress. Cells undergoing apoptosis show characteristic morphological and biochemical features, which can be readily measured and quantified. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed "secondary necrosis".

As used herein, the terms "**cell**," "**cells**," "**cell line**," "**host cell**," and "**host cells**," are used interchangeably and, encompass plant, and animal cells and include invertebrate, non-mammalian vertebrate and mammalian cells. All such designations include cell populations and progeny. Thus, the terms "transformants" and "transfectants" include the primary subject cell and cell lines derived therefrom without regard for the number of transfers. Exemplary non-mammalian vertebrate cells include, for example, avian cells, reptilian cells and amphibian cells.

Exemplary invertebrate cells include, but are not limited to, insect cells such as, for example, caterpillar (Spodoptera frugiperda) cells, mosquito (Aedes aegypti) cells, fruitfly (Drosophila melanogaster) cells, Schneider cells, and Bombyx mori cells. See, e.g., Luckow et al., Bio/Technology 6:47-55 (1988) The cells may be differentiated, partially differentiated or undifferentiated, e.g. stem cells, including embryonic stem cells and pluripotent stem cells. Additionally tissue samples derived from organs or organ systems may be used according to the invention. Exemplary mammalian cells include, for example, cells derived from human, non-human primate, cat, dog, sheep, goat, cow, horse, pig, rabbit, rodents including mouse, hamster, rat and guinea pig and any derivatives and progenies thereof

The terms "**cell culture**," or "**tissue culture**" refer to cells grown in suspension or grown adhered to a variety of surfaces or substrates in vessels such as roller bottles, tissue culture flasks, dishes, multi-well plates and the like. Large scale approaches, such as bioreactors, including adherent cells growing attached to microcarriers in stirred fermentors, are also encompassed by the term "cell culture." Moreover, it is possible not only to culture contact-dependent cells, but also to use suspension culture techniques in the methods of the claimed invention. Exemplary microcarriers include, for example, dextran, collagen, plastic, gelatin and cellulose and others as described in Butler, Spier & Griffiths, Animal cell Biotechnology 3:283-303 (1988). Porous carriers, such as, for example, Cytoline™ or Cytopore™, as well as dextran-based carriers, such as DEAE-dextran (Cytodex 1™ quaternary amine-coated dextran (Cytodex™) or gelatin-based carriers, such as gelatin-coated dextran (Cytodex 3™) may also be used. Cell culture procedures for both large and small-scale production of proteins are encompassed by the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, with or without microcarriers, and operated alternatively in a batch, fed-batch, or perfusion mode.

The terms "**cell culture medium**," "**cell culture media**," and "**culture medium**" refer to the solutions used for growing, storing, handling and maintaining cells and cell lines. Such solutions generally include various factors necessary for cell attachment, growth, and maintenance of the cellular environment. For example, a typical solution may include a basal media formulation, various supplements depending on the cell type and, occasionally, antibiotics. In some embodiments, a solution may include at least one component from one or more of the following categories. 1) an energy source, usually in the form of a carbohydrate such as glucose; 2) all essential amino acids, and usually the basic set of twenty amino acids plus cystine; 3) vitamins and/or other organic compounds required at low concentrations; 4) free fatty acids; and 5) trace

elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range. The solution may optionally be supplemented with one or more components from any of the following categories: 1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor; 2) salts and buffers as, for example, calcium, magnesium, phosphate, Tris, HEPES, and sodium bicarbonate; 3) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and 4) protein and tissue hydrolysates. In general, any suitable cell culture medium may be used. The medium may be comprised of serum, e.g. fetal bovine serum, calf serum or the like. Alternatively, the medium may be serum free, animal free, or protein free.

The term "**cell culture media component**" or "cell culture component" refers to any heterologous proteins used as a supplement for cell culture media.

The term "**cell culture media ingredient**" includes Cell culture media components, proteins, peptides, hormones, carbohydrates, amino acids, lipids, vitamins, antibiotics, organic and inorganic salts.

The term "**cell culture media supplement**" refers to a combination of one or multiple cell culture media components, with or without other ingredients, for addition to cell culture media.

The term "**cell culture media base**" or "**basal media**" refers to a cell culture media that may contain, for example, any or all of the following components: proteins, peptides, lipids, carbohydrates, amino acids, organic and/or inorganic salts, buffers (e.g., bicarbonate), vitamins, hormones, antibiotics, and pH indicators (e.g., phenol red). Examples of cell culture media bases that can be used in accordance with the present invention include: Dulbecco's Modified Eagle's Medium (DME), Ham's Nutrient Mixtures, MCDB Media, Minimum Essential Medium Eagle, RPMI Media, Ames' Media, BGJb Medium (Fitton-Jackson Modification), Click's Medium, CMRL-1066 Medium, Fischer's Medium, Glascow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), L-15 Medium (Leibovitz), McCoy's 5A Modified Medium, NCTC Medium, Swim's S-77 Medium, Waymouth Medium, and William's Medium E. The cell culture media base may be supplemented using a cell culture media component or a cell culture media supplement.

The term "**cell lineage**" when referring to a stem cell culture refers to all of the stages of the development of a cell type, from the earliest precursor cell to a completely mature cell (i.e. a

specialized cell).

The terms "**cell viability**" or "**viability**" refers to relative amounts of living and dead cells, present with a population of cells at any given time Cell viability may be determined by measuring the relative numbers of living and dead cells in any given sample of the population. Cell viability may also be estimated by measuring the rate of cell proliferation of the entire population which represents the overall balance of the rates of cell growth and cell death. Rates of cell growth may also be directly measured, by counting the number of cells, and by using any number of commercially available cell proliferation assays which directly scores the rate of cell growth.

"**Conditioned medium**" refers to a cell culture medium that is obtained from a culture of a feeder cell on which embryonic stem cells can be cultured and maintained in a pluripotent state. The feeder cell depletes the conditioned medium of some components, but also enriches the medium with cell-derived material, probably including small amounts of growth factors. The term "feeder cell factor" as used herein means the cell-derived material that is released into the conditioned medium by the feeder cell. The cell factor that is released into the cell culture medium is useful in enhancing the growth of embryonic stem cells, or in the maintenance of the embryonic stem cell in a pluripotent state. The feeder cell factor can be identified and purified using techniques that are known to one skilled in the art, and are described herein

The phrase "**conservative amino acid substitution**" or "**conservative mutation**" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag)

Examples of amino acid groups defined in this manner include: a "charged / polar group," consisting of Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic, or cyclic group," consisting of Pro, Phe, Tyr and Trp; and an "aliphatic group" consisting of Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys

Within each group, subgroups can also be identified, for example, the group of charged / polar

amino acids can be sub-divided into the sub-groups consisting of the "positively-charged sub-group," consisting of Lys, Arg and His; the negatively-charged sub-group," consisting of Glu and Asp, and the "polar sub-group" consisting of Asn and Gln. The aromatic or cyclic group can be sub-divided into the sub-groups consisting of the "nitrogen ring sub-group," consisting of Pro, His and Trp; and the "phenyl sub-group" consisting of Phe and Tyr. The aliphatic group can be sub-divided into the sub-groups consisting of the "large aliphatic non-polar sub-group," consisting of Val, Leu and Ile; the "aliphatic slightly-polar sub-group," consisting of Met, Ser, Thr and Cys; and the "small-residue sub-group," consisting of Gly and Ala.

Examples of conservative mutations include substitutions of amino acids within the sub-groups above, for example, Lys for Arg and vice versa such that a positive charge can be maintained; Glu for Asp and vice versa such that a negative charge can be maintained; Ser for Thr such that a free -OH can be maintained, and Gln for Asn such that a free -NH_2 can be maintained.

The term "**cytotoxicity**" refers to the cell killing property of a chemical compound (such as a chemical or protein contaminant, detergent, or toxin). In contrast to necrosis and apoptosis, the term cytotoxicity need not necessarily indicate a specific cellular death mechanism.

As used herein, the term "**decrease**" or the related terms "**decreased**," "**reduce**" or "**reduced**" refers to a statistically significant decrease. For the avoidance of doubt, the terms generally refer to at least a 10% decrease in a given parameter, and can encompass at least a 20% decrease, 30% decrease, 40% decrease, 50% decrease, 60% decrease, 70% decrease, 80% decrease, 90% decrease, 95% decrease, 97% decrease, 99% or even a 100% decrease (i.e., the measured parameter is at zero).

As used herein, the terms "**develop**", "**differentiate**" and "**mature**", as used to describe a stem cell, refer to the progression of a cell from the stage of having the potential to differentiate into at least two different cellular lineages to becoming a specialized and terminally differentiated cell. Such terms can be used interchangeably for the purposes of the present application.

The term "**expression**" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, in situ hybridization to cellular RNA or by PCR. Proteins encoded

by a selected sequence can be quantified by various methods including, but not limited to, e.g., ELISA, Western blotting, radioimmunoassays, immunoprecipitation, assaying for the biological activity of the protein, or by immunostaining of the protein followed by FACS analysis.

"Expression control sequences" are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, internal ribosome entry sites (IRES) and the like, that provide for the expression of a coding sequence in a host cell. Exemplary expression control sequences are described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego Calif. (1990).

The term "**feeder cell**" refers to a culture of cells that grows in vitro and secretes at least one factor into the culture medium, and that can be used to support the growth of another cell of interest in culture. As used herein, a "**feeder cell layer**" can be used interchangeably with the term "feeder cell." A feeder cell can comprise a monolayer, where the feeder cells cover the surface of the culture dish with a complete layer before growing on top of each other, or can comprise clusters of cells

The term "**growth phase**" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are dividing at a constant rate. During this phase, cells are cultured for a period of time, and under such conditions that cell growth is maximized. The determination of the growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. "Period of time and under such conditions that cell growth is maximized" and the like, refer to those culture conditions that, for a particular cell line, are determined to be optimal for cell growth and division. During the growth phase, cells are cultured in nutrient medium containing the necessary additives usually at about 30-40 ° C., generally about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line, for instance a mammalian cell.

The term "**heterologous DNA**" refers to DNA which has been introduced into a cell that is derived from another source, or which is from the same source but is located in a different (i.e. non native) context. The term "**heterologous protein**" or "**recombinant protein**" refers to a protein encoded in all or part by heterologous DNA, or a protein that is expressed from expression control sequences (such as a promoter, or enhancer) created in whole or part by the heterologous DNA which activates the expression of an endogenous gene.

The term "**homology**" describes a mathematically based comparison of sequence similarities

which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes. Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used.

The term "**homologous**" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (e.g., the immunoglobulin superfamily) in the same species of animal, as well as homologous proteins from different species of animal (for example, myosin light chain polypeptide, etc., see Reeck et al., *Cell*, 50:667, 1987). Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term "**growth factor**" refers to Amphiregulin, Angiopoietin, Betacellulin, (Bone Morphogenic protein-13, Bone Morphogenic protein-14, Bone Morphogenic protein-2, Human BMP-3, Bone Morphogenic protein-4, Human BMP-5, Bone Morphogenic protein-6, Bone Morphogenic protein-7, Human CD135 Ligand / Flt-3 Ligand, Human Granulocyte Colony Stimulating Factor (G-CSF), Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Human Macrophage Colony Stimulating Factor (M-CSF), Human Cripto-1, Human CTGF (Connective tissue growth factor), Human EGF (Epidermal Growth Factor), Human EG-VEGF (Endocrine-Gland-Derived Vascular Endothelial Growth Factor), Human Erythropoietin (EPO), Human FGF (Fibroblast Growth Factors 1-23), Human GDF-11, Human GDF-15, Human GDF-8, Human Growth Hormone Releasing Factor (GHRF, GRF, GHRH, Growth Hormone Releasing Hormone), Human Heparin Binding Epidermal Growth Factor (HB-EGF), Human Hepatocyte Growth Factor (HGF), Human Heregulin beta 1, Human insulin, Human IGF-1 (Insulin-like Growth Factor-1), Human IGF-2 (Insulin-like Growth Factor-2), Human IGFBP-1 (Insulin-like Growth Factor Binding Protein 1), Human IGFBP-3 (Insulin-like Growth Factor Binding Protein 3), intestinal trefoil factor (ITF), Human keratinocyte growth factors 1 & 2, Human Leukemia

Inhibitory Factor (LIF), Human MSP, Human Myostatin, Human Myostatin, pro (propeptide), Human NRG1, Human NGF, Human Oncostatin M, Human Osteoblast Specific Factor 1 (OSF-1, Pleiotrophin), Human PD-ECGF (Platelet-derived endothelial cell growth factor), Human PDGF, Human PlGF, Human Placental Growth Factor 1 (PLGF1), Human Placental Growth Factor 2 (PLGF2), Human SCGF-a (Stem Cell Growth Factor-alpha), Human SCGF-b (Stem Cell Growth Factor-beta), Human Stem Cell Factor (SCF) / CD117 Ligand, Human Thrombopoietin (TPO, THPO), Human Transforming Growth Factor, Human TGF-alpha (Transforming Growth Factor-alpha, TGFa), Human TGF-beta 1 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 1.2 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 2 (Transforming Growth Factor-beta2, TGFb), Human TGF-beta 3 (Transforming Growth Factor-beta3, TGFb), Human VEGF (Vascular Endothelial Growth Factor), Human VEGF-121, Human VEGF-165, and Human VEGF-A

As used herein, "**identity**" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds , Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991, and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm can also be used to determine identity.

The terms "**immunoglobulin**" or "**antibody**" (used interchangeably herein) refers to a protein typically having a basic four-polypeptide chain structure consisting of two heavy and two light chains, the chains being stabilized, for example, by interchain disulfide bonds, which has the

ability to specifically bind antigen. The term "single-chain immunoglobulin" or "single-chain antibody" (used interchangeably herein) refers to a protein having a two-polypeptide chain structure consisting of a heavy and a light chain, the chains being stabilized, for example, by interchain peptide linkers, which has the ability to specifically bind antigen. The term "domain" refers to a globular region of a heavy or light chain polypeptide comprising peptide loops (e.g., comprising 3 to 4 peptide loops) stabilized, for example, by beta-pleated sheet and/or intrachain disulfide bond. Domains are further referred to herein as "constant" or "variable", based on the relative lack of sequence variation within the domains of various class members in the case of a "constant" domain, or the significant variation within the domains of various class members in the case of a "variable" domain. Antibody or polypeptide "domains" are often referred to interchangeably in the art as antibody or polypeptide "regions". The "constant" domains of an antibody light chain are referred to interchangeably as "light chain constant regions", "light chain constant domains", "CL" regions or "CL" domains. The "constant" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "CH" regions or "CH" domains). The "variable" domains of an antibody light chain are referred to interchangeably as "light chain variable regions", "light chain variable domains", "VL" regions or "VL" domains). The "variable" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "VH" regions or "VH" domains). Immunoglobulins or antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Exemplary fragments include Fab, Fab', F(ab')2, Fabc and/or Fv fragments.

As used herein, the term "**increase**" or the related terms "**increased**", "**enhance**" or "**enhanced**" refers to a statistically significant increase. For the avoidance of doubt, the terms generally refer to at least a 10% increase in a given parameter, and can encompass at least a 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 95% increase, 97% increase, 99% or even a 100% increase over the control value.

The term "**isolated**," when used to describe the cell culture components, i.e. albumin, or transferrin related proteins disclosed herein, means protein that has been identified and

separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily however, isolated protein will be prepared by at least one purification step

The term "**maturation-specific protein promoter**" refers to a promoter exhibiting substantially up-regulated activity (greater than 25%) during seed maturation.

The term "**mature plant**" refers to a fully differentiated plant.

"**Markers**" as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

Cells expressing "**markers of pancreatic endocrine lineage**" refer to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, NRx2.2, NRx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, and Pax6. Cells expressing markers characteristic of the pancreatic cell lineage include pancreatic β cells.

Cells expressing "**markers characteristic of endoderm lineage**" as used herein refer to cells expressing at least one of the following markers: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA-6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

Cells expressing pluripotency markers derived by the methods of the present invention express at least one of the following pluripotency markers selected from the group consisting of: ABCG2,

cripto, FoxD3, Connexin43, Connexin45, Oct4, SOX-2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tral-60, and Tral-81.

Cells expressing "**markers characteristic of mesoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, GATA-6.

Cells expressing "**markers characteristics of ectoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: BMP-4, Noggin, Chordin, Otx2, Fox J3, Nestin, p63/TP73L, beta-III Tubulin.

"**Monocot seed components**" refers to carbohydrate, protein, and lipid components extractable from monocot seeds, typically mature monocot seeds

As used herein, the terms "**native**" or "**wild-type**" relative to a given cell, protein, polypeptide, nucleic acid, trait or phenotype, refers to the form in which that is typically found in nature.

The terms "**operably linked**" and "**operatively linked**," as used interchangeably herein, refer to the positioning of two or more nucleotide sequences or sequence elements in a manner which permits them to function in their intended manner. In some embodiments, a nucleic acid molecule according to the invention includes one or more DNA elements capable of opening chromatin and/or maintaining chromatin in an open state operably linked to a nucleotide sequence encoding a recombinant protein. In other embodiments, a nucleic acid molecule may additionally include one or more nucleotide sequences chosen from: (a) a nucleotide sequence capable of increasing translation; (b) a nucleotide sequence capable of increasing secretion of the recombinant protein outside a cell; and (c) a nucleotide sequence capable of increasing the mRNA stability, where such nucleotide sequences are operatively linked to a nucleotide sequence encoding a recombinant protein. Generally, but not necessarily, the nucleotide sequences that are operably linked are contiguous and, where necessary, in reading frame. However, although an operably linked DNA element capable of opening chromatin and/or maintaining chromatin in an open state is generally located upstream of a nucleotide sequence encoding a recombinant protein; it is not necessarily contiguous with it. Operable linking of various nucleotide sequences is accomplished by recombinant methods well known in the art, e.g using PCR methodology, by ligation at suitable restrictions sites or by annealing. Synthetic oligonucleotide linkers or adaptors can be used in accord with conventional practice if suitable restriction sites are not present.

A "**plant cell**" refers to any cell derived from a plant, including undifferentiated tissue (e.g., callus) as well as plant seeds, pollen, propagules, embryos, suspension cultures, meristematic regions, leaves, roots, shoots, gametophytes, sporophytes and microspores.

The terms "**polynucleotide**" and "**nucleic acid molecule**," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. A nucleic acid molecule can take many different forms, e.g., a gene or gene fragment, one or more exons, one or more introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. As used herein, "DNA" or "nucleotide sequence" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides

The term "**pluripotent stem cell**" encompasses stem cells obtained from embryos, fetuses or adult tissues. In one preferred embodiment, the pluripotent stem cell is an embryonic stem cell. In another embodiment the pluripotent stem cell is a fetal stem cell, such as a primordial germ cell. In another embodiment the pluripotent stem cell is an adult stem cell.

As used herein, the term "**pluripotent**" refers to a cell capable of at least developing into one of ectodermal, endodermal and mesodermal cells. As used herein the term "pluripotent" includes cells that are totipotent and multipotent. As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. The term "multipotent" refers to a cell that is not terminally differentiated.

A "**promoter**" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. As used herein, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. A transcription initiation site (conveniently defined by mapping with nuclease S1) can be found within a promoter sequence, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters can often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine- Dalgarno sequences in addition to the -10 and -35 consensus sequences.

A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources are well known in the art. Representative sources include for example, viral, mammalian, insect, plant, yeast, and bacterial cell types, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available on line or, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, the RSV promoter, the rice endosperm specific glutelin (Gt1) promoter, CaMV35S viral promoter. Inducible promoters include the Tet system, (US Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., Proc. Natl. Acad. Sci. (1996) 93 (8): 3346-3351; the T-RE$_x$™ system (Invitrogen Carlsbad, CA), LacSwitch® (Stratagene, (San Diego, CA) and the Cre-ER™ tamoxifen inducible recombinase system (Indra et al. Nuc. Acid. Res. (1999) 27 (22): 4324-4327; Nuc. Acid. Res. (2000) 28 (23): e99; US Patent No. 7,112,715; and Kramer & Fussenegger, Methods Mol. Biol. (2005) 308. 123-144) or any promoter known in the art suitable for expression in the desired cells.

The term "**protein of interest**" refers to any protein which may be useful for research, diagnostic or therapeutic purposes. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. Exemplary proteins of interest include, but are not limited to, molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin;

insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor, lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; members of the TNF and TNF receptor (TNFR) family, like tumor necrosis factor-alpha and -beta, CD40 ligand, Apo-2 ligand/TRAIL, DR4, DR5, DcR1, DcR2, DcR3, OPG, Fas ligand; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide, a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D, rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TG-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20, erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma, colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; thrombopoietin (TPO); interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope, gp120; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and variants and/or fragments of any of the above-listed polypeptides; as well as antibodies against various protein antigens like CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34, members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, an Apo-2L receptor such as Apo-2 (DR5), DR4, DcR1, DcR2, DcR3; and variants and/or fragments of the above-identified antibodies etc. In one embodiment of the invention, a protein of interest will comprise a protein which itself is capable

of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

The term "**production phase**" of the cell culture refers to the period of time during which cell growth has reached a plateau. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product

The term "**produced in a plant**" when used to describe the cell culture components disclosed herein. means that the protein has been obtained directly or indirectly from a plant cell. With respect to recombinant proteins, the term "produced in a plant" means that the recombinant protein has been expressed within a plant cell, and then purified from host cells proteins and lipids. Proteins that have been produced in a plant may be differentiated from proteins that have purified from other organisms by at least three criteria. 1) Proteins produced in a plant will typically comprise different patterns of post-translational modifications compared to proteins produced in different organisms, which may be readily identified by mass spectrum analysis 2) Proteins produced in a plant will typically comprise a different set of bound co-factors, lipids and contaminating proteins compared to the same protein produced in a different expression system, or purified from a different source. 3) Proteins produced in a plant may exhibit a different biological activity compared to the same protein produced or purified from a different source. The difference in activity in the protein produced in a plant may arise from a difference in the conformation, folding, aggregation state, post-translational modifications, or bound factors or co-purified contaminating proteins of the protein produced in a plant compared to a protein produced or isolated from a different organism. Differences in the specific activity of a protein produced in a plant may be readily established by routine assays using defined concentrations and assay conditions, for example, using cell growth, and factor synergism assays, as disclosed in Examples 11 to 19. Specifically a protein produced in a plant may exhibit at least at least 10 % greater cell growth activity either alone, or in combination with other factors compared to the same concentration of a non plant produced protein. Preferably, a protein produced in a plant may exhibit at least at least 20 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 30 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 40 % greater cell growth activity either alone, or in combination with other factors,

compared to the same concentration of a non plant produced protein. Even more preferably, a protein produced in a plant may exhibit at least at least 50 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein.

The terms "**isolated**" or "**purified**" when used to describe the cell culture components disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step

The term "**recombinant protein**" or "**recombinant polypeptide**" refers to an exogenous, i.e., heterologous or foreign polypeptide, to the cells producing the polypeptide.

The term "**stress**" in the context of apoptosis or cell culture refers to non-optimal conditions for tissue culture including any combination of the following; the presence of toxins, nutrient or growth factor depletion or withdrawal, hypoxia, thermal stress (temperature is too high or too low compared to the preferred range), loss of cell-cell contacts, viral infection, osmotic stress (osmolality is too high or too low compared to the preferred range), oxidative stress, cell density (cell density is too high or too low compared to the preferred range), and pH stress (pH is too high or too low compared to the preferred range)

The term "**transformation**" refers to the transfer of one or more nucleic acid molecules into a host cell or organism. Methods of introducing nucleic acid molecules into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, scrape loading, ballistic introduction or infection with viruses or other infectious agents. "**Transformed**", "**transduced**", "**transgenic**", and "**recombinant**" refer to a host cell or organism into which a recombinant or heterologous nucleic acid molecule (e.g., one or more DNA constructs or RNA, or siRNA counterparts) has been introduced. The nucleic acid molecule can be stably expressed (i.e maintained in a functional form in the cell for longer than about three months) or non-stably maintained in a

functional form in the cell for less than three months i.e. is transiently expressed. For example, "transformed," "transformant," and "transgenic" cells have been through the transformation process and contain foreign nucleic acid. The term "untransformed" refers to cells that have not been through the transformation process.

The term "**transition phase**" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as pH, ion concentration, and temperature may shift from growth conditions to production conditions.

The term "**seed product**" includes, but is not limited to, seed fractions such as de-hulled whole seed, flour (seed that has been de-hulled by milling and ground into a powder) a seed extract, preferably a protein extract (where the protein fraction of the flour has been separated from the carbohydrate fraction), malt (including malt extract or malt syrup) and/or a purified protein fraction derived from the transgenic grain.

"**Seed maturation**" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

The term "**sequence similarity**" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (see Reeck et al., supra). However, in common usage and in the instant application, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 85%, and more preferably at least about 90% or at least about 95% of the nucleotides match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm known such as BLAST, FASTA, DNA Strider, CLUSTAL, etc. An example of such a sequence is an allelic or species variant of the specific genes of the present invention. Sequences that are substantially homologous may also be identified by hybridization, e.g., in a Southern hybridization experiment under, e.g., stringent conditions as defined for that particular system.

Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acid residues are identical, or when greater than about 90% of the amino acid residues are similar (i.e., are functionally identical). Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above. The program may use the local homology algorithm of Smith and Waterman with the default values: Gap creation penalty = -(1+1/*k*), *k* being the gap extension number, Average match = 1, Average mismatch = -0.333.

As used herein and in the appended claims, the singular forms "a," "an," and "the," include plural referents unless the context clearly indicates otherwise. Thus, for example reference to "a molecule" includes one or more of such molecules, "a reagent" includes one or more of such different reagents, reference to "an antibody" includes one or more of such different antibodies, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, miocrobiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands;. DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990. In Situ Hybridization: Principles and Practice; Oxford University Press. M J. Gait (Editor), 1984, Oligonucleotide Synthesis. A Practical Approach, Irl Press, D. M J Lilley and J. E. Dahlberg, 1992, Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press; Using Antibodies: A Laboratory Manual: Portable Protocol NO I by Edward Harlow, David Lane. Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7), Antibodies: A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-3,4-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, N.Y , Marcel

Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods, compositions, reagents, cells, similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described herein. All publications mentioned herein are incorporated herein, including all figures, graphs, equations, illustrations, and drawings, to describe and disclose specific information for which the reference was cited in connection with.

I. Methods of using the supplements of the invention

Frequently, cell culture media is supplemented with serum at concentration of 5 to 10%. Serum is a complex mixture containing undefined essential matter for cell growth. Commonly used serum is fetal calf serum (FCS) and fetal bovine serum (FBS). Due to its complex nature, lack of consistency, and the potential risk for pathogen contamination, cell culture industries and regulatory agencies are seeking alternatives to adding serum to cell culture media.

Cell culture components and cell culture supplements added to basal media serve distinct functions in promoting cell growth. These protein components are typically animal-derived. With the increase in the concern of potential pathogen contamination from animal sourced material, cell culture industries are trying to develop animal-component-free media. While some successes have been made with the use of plant-based hydrolysates and recombinant proteins produced using microbial systems, plant-based hydrolysates are an undefined mixture of components and recombinant protein from microbial sources are too expensive to use in routine cell culture. An alternative source of non-animal-derived components would be beneficial for industries that depend on cell culture techniques.

Accordingly in one embodiment the instant invention provides a method for culturing cells,

including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

According to further embodiments, the combination of proteins that is used in the methods of culturing cells and supplements of the invention provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, the supplements comprising one combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

In another aspect, the supplements comprises a combination of transferrin related proteins and albumin which provide a synergistic effect on cell growth and productivity.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, adding a supplement of the present invention to the cell culture medium.

The claimed supplements are useful in a wide range of applications for tissue and cell culture and recombinant protein production where they provide for significant improvements in preventing apoptosis and improving cell viability during tissue culture, and in particular in response to stress

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. These changes include, changes to the cell membrane such as loss of membrane asymmetry and attachment, cellular blebbing cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, cytokines, which may be present to different degrees in tissue culture media. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis, and thus influence overall cell viability A number of intracellular components, including ATP content, calcium level, and a number of apoptotic and anti-apoptotic genes also help regulate apoptosis. A cell may initiate intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. Stress inducing agents encountered during tissue culture include for example toxins, associated with tissue culture components such as endotoxins, and heavy metals that leach from plastic ware, transfection reagents (e.g Lipofectamine and similar lipid based transfection reagents), viral transformation, nutrient and growth factor deprivation, associated with serum free culture, or cell differentiation protocols hypoxia and oxidative stress associated with high density culture in a bioreactor and increased intracellular calcium concentration, for example, by damage to the membrane caused by detergents and electroporation.

Before the actual process of cell death occurs, the apoptotic signals must overcome regulatory proteins which act as gatekeepers overseeing the activation of the apoptosis pathway. In vivo, this step allows the process to be stopped, should the cell no longer need to die. Several proteins are involved at this step, though two main mechanisms of regulation have been identified and include those associated with mitochondria functionality, and those directly involved in transducing the signal via adaptor proteins to the apoptotic mechanisms.

Cells grown under cell culture conditions may experience cellular stresses associated with routine tissue culture procedures, as described above which may trigger apoptotic signals and increase the susceptibility of the cells to apoptosis. For example, nutrient deprivation associated with serum free culture, oxidative stress associated with high density growth in a bioreactor, the use of cytotoxic compounds associated with DNA transfection reagents, and thermal stresses associated with cryopreservation, may predispose the cell to enter apoptosis. By enhancing the ability of a cell to survive such signals it is possible to improve cell viability

during these procedures. by preventing the cells commitment to cell death, thereby improving the success and utility of these approaches.

Recently a number of genes in eukaryotic cells have been identified which inhibit the onset or reduce the effects of apoptosis. Some of these genes inhibit caspase dependent apoptotic pathways in the cell, and in fact transfecting cells with anti-apoptotic genes may be useful in prolonging the life and productivity of transfected cells grown under biologically demanding conditions (US Patent Nos., 6,586,206; 7,531,327, US Patent Application US 2009/0170165; US2009/0181426).

Additionally the addition of exogenous heat shock proteins has in some cases been shown to improve the survival of cells in culture under a variety of conditions. (Novoselova et al., J. Neurochem. 94 597-606 (2005); Tidwell et al., Cell Stress & Chap 9 (1) 88-90 (2004); Guzhova et al., Cell Stress & Chap. 3 (1) 67-77 (1998); Hounenou et al., Cell Stress & Chap 1 (3) 161-166 (1996); Johnson et al , In vitro Cell. Dev. Biol., 29A 807-812 (1993)

The present invention is based in part on the demonstration that plant derived recombinant cell culture component proteins surprisingly enhanced the cell growth and viability when added in specific mixtures to mammalian cells grown in culture. Surprisingly some combinations of proteins provide for a synergistic effect on cell growth and productivity which provides unexpected benefits compared to the use of the individual proteins Specifically, such supplements comprising plant derived recombinant albumin and transferrin related proteins result in improved culture viability, extended cell survival, improved rates of cell growth and improved yields of recombinant proteins produced from tissue culture bioreactors. Because the supplements show unexpectedly improved activity and stability they offer significant improvements compared to the use of standard recombinant or purified proteins.

The supplements disclosed in the present application are useful, for example, for improving cell viability and in accelerating the rate of cell growth of cells grown in culture. In one aspect, the supplements of the invention are useful for improving or enhancing the yield of the recombinant proteins from the cell cultures. Further improvements provided by the invention are described in detail below.

In one embodiment, the present invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium

In one embodiment. the present invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media

In one embodiment, the present invention includes a method for reducing the accumulation of lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment. the present invention includes a method of reducing time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor

In one embodiment, the present invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium.

In one embodiment, the present invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium

In one embodiment, the present invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection

In one embodiment, the present invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing.

In one embodiment, the present invention includes a method for improving the rate of cell growth or viability of stem cells grown in culture comprising the addition of a supplement of the present invention to the cell culture media.

In one embodiment, the present invention includes a method for improving the yield of a recombinant product produced from cells in culture comprising the addition of a supplement of the present invention to the cell culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the purification of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for reducing the proteolysis of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the bioactivity of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the stability of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the assembly of a recombinant product produced from cells in culture, comprising the addition of a supplement to

the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a more human pattern of glycosylation of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a recombinant product produced from cells in culture with less immunogenicity, comprising the addition of a supplement comprising recombinant albumin to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In any of these methods, the supplements of the invention, by increasing host cell viability in culture (and during fermentation), provide for a simple and cost effective method to increase the yield, and or purity, bioactivity, stability and assembly of functional recombinant protein Additionally, the supplements of the invention, by decreasing or inhibiting apoptosis in the cell culture, can decrease the number or presence of adverse proteases in the culture media and protect the expressed protein of interest against proteolytic degradation, thereby increasing the quality of the protein of interest produced, as evidenced by increased amounts of active protein, and increased yields of intact protein. Additionally Applicants have found that the supplements of the invention may protect the cells against potential adverse effects of agents like detergents, heavy metals and endotoxin contaminates present in the culture components, or protect the cells from toxic reagents introduced to the cells during transfection or cryopreservation.

In any of the claimed methods, the supplements of the invention can be added directly, or admixed, to the culture media at any convenient time, for example when changing the media, passaging the cells, or when plating out the cells at low density. Optionally, the supplement is added to the culture media at the beginning (at the time of initiating, day 0) of the cell culturing process. In one aspect the supplements of the invention may be added before an anticipated stressful event, for example before cryopreservation, transfection or serum withdrawal, etc.

In another aspect, the supplement is added to the culture media during the culturing of the cells prior to the point when induction of typically apoptosis occurs For example, during a large scale cell culture, induction of apoptosis can be observed on about day 3 or day 4 of the culture, and therefore, the supplement will preferably be added prior to day 3 or day 4. Optionally, a

desired quantity of the supplement is added throughout, or for the duration of, the cell culture, for instance, on a daily basis for the entire fermentation. As an example, for a 5 day culture, the supplement could be added at day 0, and every 24 hours thereafter until the culture is terminated.

Accordingly in one embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a bioreactor by adding a supplement of the invention to the bioreactor. In one embodiment, the bioreactor comprises bacterial cells. In another aspect the bioreactor comprises yeast cells. In another aspect the bioreactor comprises plant cells. In another aspect the bioreactor comprises mammalian cells

In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in bacterial cells, by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in yeast cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a plant cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in insect cells by adding the supplement of the invention to the cell culture. In another embodiment the invention provides a method of improving the yield and quality of a recombinant protein produced in mammalian cells by adding the supplement of the invention to the cell culture.

In another embodiment, the invention provides a method to increase the yield of the production phase of a cell culture system and thereby increase the productivity of a bioreactor by adding the supplement of the invention to the cell culture system prior to, or during the production phase of the cell culture system.

In one aspect of this method the yield of the production phase is increased by about 10%. In one aspect of this method the yield of the production phase is increased by about 20%. In one aspect of this method the yield of the production phase is increased by about 30%. In one aspect of this method the yield of the production phase is increased by about 40%. In one aspect of this method the yield of the production phase is increased by about 50%. In one aspect of this method the yield of the production phase is increased by about 60%. In one aspect of this method the yield of the production phase is increased by about 70%. In one aspect of this method the yield of the production phase is increased by about 80%. In one

aspect of this method the yield of the production phase is increased by about 90%. In one aspect of this method the yield of the production phase is increased by about 100%. In one aspect of this method the yield of the production phase is increased by about 200%. In one aspect of this method the yield of the production phase is increased by about 500%.

In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which the supplement was not added. In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which is added a supplement lacking either a transferrin related protein, or recombinant serum albumin. In one aspect, the yield is increased compared to the summed effect of the recombinant albumin and the transferrin related protein when added alone when measured under the same culture conditions

In another embodiment, the invention provides a method to produce a protein of interest at a temperature that is elevated compared to normal growth conditions for the production of that protein, comprising the addition of a supplement of the invention to cells expressing the protein of interest.

In another embodiment, the invention provides a method to decrease the amount of aggregates formed in a cell culture expression system by aggregate prone proteins of interest comprising the addition of a supplement of the invention to the cell culture expression system, whereby the aggregation state of the protein is reduced.

In another embodiment, the invention provides a method to increase the activity of a protein of interest protein expressed by a cell by preventing the denaturation and aggregation of the recombinant protein comprising the addition of a supplement of the invention to the cell, whereby the specific activity of the protein of interest is increased.

In another embodiment, the invention provides a method to improve the expression of proteins in a cell culture expression system that are aggregation prone, cause precipitation to occur, or are toxic themselves to the cells comprising the addition of a supplement of the invention to the cell culture expression system, whereby the expression of the protein of interest is increased.

The amount of supplement to add in any of these methods will depend on various factors, for instance, the type of host cell, the cell density, protein of interest and culture conditions, etc Determining the desired concentration of supplement to be added to the culture media is within the skill in the art and can be ascertained empirically by routine optimization and without undue experimentation.

The skilled artisan will readily appreciate that different cell types will have different magnitudes of responses to the supplement of the invention, and this will be determined, to some degree, by the amount or type of the proteins in the supplement. Additionally different densities of cells will require appropriate adjustment in the total amount of supplement as well as the concentration of the individual proteins added to the culture to account for the increased cell number. Additionally cells grown in suspension culture or via adherent culture will have different membrane surface areas available for protein entry and will typically exhibit different rates and degrees of response. Therefore, one should choose a concentration which provides for a sufficient inhibition of apoptosis, or increase in viability, or net cell growth. Typically the supplements of the invention will be added to a final concentration of about 0.1 %, about 0.5 %, about 1%, about 2%, about 3 %, about 4 %, about 5 %, about 6%, about 7%, about 8%, about 9%, about 10 %, about 11 %, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 25%, about 30%, about 40%, or about 50% wt/wt, or wt /volume

There will typicelly be an upper range of concentration of the supplement beyond which further increases in cell survival do not occur. As described in the Examples below, Applicants have found that the supplements of the invention can inhibit apoptosis when added to cell cultures at a concentration of about 200 mg /L to about 2g/L, or more preferably about 200 mg/L to about 1000 mg/L, or more preferable about 250 to about 500 mg/L.

II. Supplements

The cell culture media, methods for producing supplemented cell culture media, and methods of culturing cells using fortified cell culture media in accordance with the present invention may include components derived from animal, plant, bacterial, yeast, and insect sources. According to one aspect of the invention, the components are derived from plant sources, and preferably from monocot plants. The components may be combined with inorganic salts such as NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, and $MgCl_2$ and other ingredients such as amino acids, vitamins, growth factors, sugars, and antibiotics to form a variety of different cell culture media. According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media

are plant-produced heterologous proteins.

According to another aspect, one of the proteins is albumin, and at least one additional protein is selected from the group consisting of growth factors, lactoferrin transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof

According to further embodiments, the combination of proteins that is used in the methods of producing the supplemented cell culture media provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, one combination of proteins that may be used in the methods of producing the cell culture media includes a combination of albumin with at least one other protein selected from the group consisting of lactoferrin, transferrin, melanotransferrin, and ovotransferin.

In one aspect the supplements of the invention comprise plant derived recombinant human serum albumin.

The term **"albumin"**, refers to all naturally-occurring and synthetic forms of albumin. Preferably, the term "albumin" refers to recombinant albumin. In one aspect the albumin is from a vertebrate. In one aspect the albumin is from a mammal. In a further embodiment the albumin is human. In another aspect, the recombinant albumin is produced from a plant cell. In one particularly preferred embodiment the recombinant albumin is produced from transgenic rice (Oryza sativa) Representative species and Gene bank accession numbers for various species of albumin are listed below in **Table D 1.**

Table D1

Exemplary Albumin genes

Species	Gene Bank Accession number
Human	NP_000468.1
Pan troglodytes	XP_517233.2
Canis lupus familiaris	XP_855557.1
Bos taurus	NP_851335.1
Mus musculus	NP_033784.1

Rattus norvegicus	NP_599153.1
Gallus gallus	NP_990592.1

It will be understood that for the recombinant production of albumin in different species it will typically be necessary to codon optimize the nucleic acid sequence of the gene for the host organism in question. Such codon optimization can be completed by standard analysis of the preferred codon usage for the host organism in question, and the synthesis of an optimized nucleic acid via standard DNA synthesis. A number of companies provide such services on a fee for services basis and include for example, DNA2.0, (CA, USA) and Operon Technologies, (CA, USA).

The albumin may be in its native form, i.e., as different allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the albumin, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the albumin.

Fragments of native or synthetic albumin sequences may also have the desirable functional properties of the peptide from which they derived and may be used in any of the methods of the invention The term "fragment" as used herein thus includes fragments of albumin provided that the fragment retains the biological or therapeutically beneficial activity of the whole molecule

For example, albumin contains at least 2 high affinity multi- metal binding sites for a number of physiologically important metals ions including copper, zinc cadmium and nickel. (Carter et al , Advances in Protein Chemistry 45 153-203 (1994), Bal et al., J. Inorg Biochem 70 (1) 33-39 (1998), Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009); US Patent No. 6,787,636). Since trace amounts of these metals are typically present in the recombinant production of albumin, a significant amount of these metal ions can be become chelated to the protein. The binding of these ions, and in particular the binding of cadmium and nickel to recombinant albumin is associated with cellular toxicity of the protein when added to cells as a tissue culture component

Accordingly, in one aspect, the albumin of the present invention can comprise a fragment of albumin that includes the deletion of one or amino acids involved in the multi-metal binding sites of albumin. In one aspect the albumin fragment is created by the deletion of one or more amino acids at the N-terminus of the mature protein. In another aspect the albumin can comprise one or more deletions or mutations of any of the amino acids involved in the N-terminal metal binding site of albumin. In one aspect, the amino acids to be deleted or mutated are independently selected from the first 10 amino acids of the mature protein.

The term "derivative" as used herein thus refers to albumin sequences or fragments thereof, which have modifications as compared to the native sequence. Such modifications may be one or more amino acid deletions, additions, insertions and/or substitutions. These may be contiguous or non-contiguous. Representative variants may include those having 1 to 20, or more preferably 1 to 15, 1 to 10, or 1 to 5 amino acid substitutions, insertions, and / or deletions as compared to any of genes listed in Tables D1. The substituted amino acid may be any amino acid, particularly one of the well-known 20 conventional amino acids (Ala (A); Cys (C); Asp (D); Glu (E); Phe (F), Gly (G); His (H); Ile (I); Lys (K); Leu (L); Met (M); Asn (N); Pro (P); Gin (Q); Arg (R); Ser (S); Thr (T); Val (V); Trp (W), and Tyr (Y)). Any such variant or derivative of albumin may be used in any of the methods of the invention.

Accordingly, the albumin of the invention can comprise amino acid deletions, insertions or mutations in any of the functional binding domains of albumin. In one aspect the albumin may comprise a mutation in a binding domain of albumin. In one aspect the mutated binding domain is a domain involved in the binding of aspirin, warfarin, diazepam, digitoxin, diofibrate, ibuprofen or AZT, as outlined is US Patent No. 5,780,593, or a multimetal binding site as outlined in Blindauer et al., J. Biol Chem. 284 (34) 23116-24 (2009)

Thus, the albumin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native albumin amino acid sequences, for example, to any of the native albumin gene sequences listed in **Table D1**. Alternatively, the albumin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with albumin listed in **Table D1**. In a preferred embodiment, the albumin for use in any of the methods of the present invention is at least 80% identical to the mature secreted human serum albumin (**SEQ. ID. No. 1**) as shown underlined in the below (Swiss-Prot P02768).

MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF

EIHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP
ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF
FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV
ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR
RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE
QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV
LNQLCVLHEK TPVSDRVTKC CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL
SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
AASQAALGL

Fusion proteins of albumin to other proteins are also included, and these fusion proteins may enhance, activity, targeting, stability or potency.

Chemical modifications of the native albumin structure which retain or stabilize albumin activity or biological half-life may also be used with any of the methods described herein. Such chemical modification strategies include without limitation pegylation, glycosylation, and acylation (see Clark et al.: *J. Biol. Chem.* 271(36): 21969-21977, 1996; Roberts et al., *Adv. Drug. Deliv. Rev.* 54(4): 459-476, (2002), Felix et al., *Int J. Pept. Protein. Res.* 46(3-4): 253-264, (1995); Garber Diabetes Obes. Metab 7 (6) 666-74 (2005)) C- and N-terminal protecting groups and peptomimetic units may also be included.

Isomers of the native L-amino acids, e.g., D-amino acids may be incorporated in any of the above forms of albumin, and used in any of the methods of the invention. All such variants, derivatives, fusion proteins, or fragments of albumin are included, may be used in any of the methods claims or disclosed herein, and are subsumed under the term "albumin"

In one aspect of the supplements of the invention the albumin is recombinant human serum albumin that has been produced in a plant. In another aspect, the albumin comprises less than about 2% aggregated albumin. In another aspect the albumin comprises less than about 1 % aggregated albumin.

In one embodiment, the supplements of the invention comprise preparations of the co-purified recombinant albumin and rice hsps that are also essentially free of detergents and endotoxins which would otherwise mask or inhibit the positive impact of the hsp. In one aspect the supplements of the invention have less than about 1 EU of endotoxin, and the albumin is at least about 95 % pure. In another aspect the supplements of the invention comprise a

composition comprising recombinant albumin bound to a heat shock protein. In another aspect the supplements of the invention comprise recombinant albumin and a rice hsp70 homolog. In one aspect the rice hsp70 homolog is selected from HSP70, Bip and rice stromal protein

The terms "**heat shock protein**", "**HSP**" or "**hsp**", as used herein includes all naturally-occurring and synthetic forms of the heat shock protein super family that retain anti-apoptotic activity. Such heat shock proteins include the small heat shock proteins/HSPB family. Hsp40/DnaJ family, HSP70/ HSPA family, HSP90 / HSPC family, HSP110/ HSPH family and chapererone family, as well as peptide fragments and protein complexes of two or more heat shock proteins or nucleotide exchange factors (for example, complexes of HSP70 & HSP40) derived therefrom. The heat shock proteins may be in their native form, i.e., as different variants as they appear in nature in different species which may be viewed as functionally equivalent variants, or they may be functionally equivalent natural derivatives thereof, which may differ in their amino acid sequence, for example, by truncation (e g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical derivatives, including post-translational modifications and degradation products of the HSPs, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, oxidatized, isomenzed, and deaminated variants of the HSP.

The term "**transferrin related protein**" refers to transferrin family proteins including transferrin, lactoferrin, melanotransferrin, and ovotransferin. The crystal structures of transferrin family members across all species that have been solved to date show a high degree of structural similarity, which is not surprising since transferrin, lactoferrin and ovotransferrin share 60-80% sequence identity. (Wally and Buchanan, Biometals (2007) 20 249-262). All transferrin related proteins contain two domains of around 340 residues, which are thought to have evolved from an ancient duplication event. For serum transferrin, ovotransferrin and lactoferrin each of the duplicated lobes binds one atom of Fe (III) and one carbonate anion. With a few notable exceptions each iron atom is coordinated to four conserved amino acid residues, an aspartic acid, two tyrosines, and a histidine, while anion binding is associated with an arginine and a threonine in close proximity. (Anderson et al., (1989) J. Mol. Biol. 209 711-734).
Human transferrin and lactoferrin share over 61 % sequence identity and have similar three dimensional structures. Transferrins transport iron in the blood by picking up free ferric iron and delivering it to cells in a receptor-mediated endocytotic process in which the TF-receptor complex is internalized, iron is released in the endosome, and the complex is recycled to the cell surface where the TF is released. In contrast, no iron transport role for lactoferrin or

ovotransferrin is known. By comparison, these proteins are thought to function to prevent invading bacteria from acquiring iron, by sequestering all available iron. Human transferrin releases iron at a higher pH than does human lactoferrin which likely relates to the need for human transferrin to release iron after endocytosis into the endosome, and the need for lactoferrin to hold onto iron in low pH environments such as the stomach (Baker & Backer (2004) Biometals 17 209-216).

The term **"transferrin"** refers to all naturally-occurring and synthetic forms of transferrin. In one aspect, the term "transferrin" refers to recombinant transferrin. In one aspect, the term "transferrin" refers to plasma derived transferrin. In one aspect the transferrin is from a vertebrate. In one aspect the transferrin is from a mammal. In a further embodiment the transferrin is human. In another aspect the recombinant transferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant transferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of transferrin are listed below in **Table D2**.

Table D2

Exemplary Transferrin genes

Species	Gene Bank Accession number
Homo sapiens	NP_001054.1
Canis lupus familiaris	XP_864550.1
Bos taurus	NP_803450.2
Mus musculus	NP_598738.1
Rattus norvegicus	NP_001013128.1
Gallus gallus	NP_990635.1
Danio rerio	NP_001015057.1

The transferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the transferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the transferrin.

The transferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native transferrin amino acid sequences, for example, to any of the native transferrin gene sequences listed in **Table D2**. Alternatively, the transferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with transferrin listed in **Table D2**. In a preferred embodiment, the transferrin for use in any of the methods of the present invention is at least 80% identical to the mature human transferrin.

The term **"Lactoferrin"** refers to all naturally-occurring and synthetic forms of Lactoferrin. In one aspect, the term "Lactoferrin" refers to recombinant Lactoferrin. In one aspect, the term "Lactoferrin" refers to milk derived Lactoferrin. In one aspect the Lactoferrin is from a vertebrate. In one aspect the Lactoferrin is from a mammal. In a further embodiment the Lactoferrin is human. In another aspect the recombinant Lactoferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant Lactoferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of Lactoferrin are listed below in **Table D3.**

Table D3

Exemplary Lactoferrin genes

Species	Gene Bank Accession number
Homo sapiens	AAA59511.1
Sus scrofa	AAA31059.1
Camelus dromedarius	CAB53387.1
Bos taurus	AAA30610.1
Equus caballus	CAA09407.1

The Lactoferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of Lactoferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of Lactoferrin. Lactoferrin which may be used in any of the methods of the

invention may have amino acid sequences which are substantially homologous, or substantially similar to the native Lactoferrin amino acid sequences, for example, to any of the native Lactoferrin gene sequences listed in **Table D3**. Alternatively, the Lactoferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with Lactoferrin listed in **Table D3**. In a preferred embodiment, the Lactoferrin for use in any of the methods of the present invention is at least 80% identical to the mature human Lactoferrin.

The term "**melanotransferrin**" refers to all naturally-occurring and synthetic forms of melanotransferrin. In one aspect, the term "melanotransferrin" refers to recombinant melanotransferrin In one aspect, the term "melanotransferrin" refers to melanotransferrin purified from a cell or tissue. In one aspect the melanotransferrin is from a vertebrate. In one aspect the melanotransferrin is from a mammal. In a further embodiment the melanotransferrin is human. In another aspect the recombinant melanotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant melanotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of melanotransferrin are listed below in **Table D4.**

Table D4

Exemplary **melanotransferrin** genes

Species	Gene Bank Accession number
Homo sapiens	AAA59992.1
Oryctolagus cuniculus	NP_001075461.1
Mus musculus	NP_038928.1
Canis lupus familiaris	XP_545158.2
Gallus gallus	CAA63003.1

The term "ovotransferrin" refers to all naturally-occurring and synthetic forms of ovotransferin. In one aspect, the term "ovotransferrin" refers to recombinant ovotransferrin In one aspect the ovotransferin is from a vertebrate. In one aspect the ovotransferin is from an avian. In another aspect the recombinant ovotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant ovotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of ovotransferrin include CAA26040.1

The term "**insulin related growth factor**" **or** "IGF-1" refers to all naturally-occurring and

synthetic forms of insulin related growth factor, including IGF-1, IGF-1A, IGF-1B, IGF-2A, and IGF-2B. In one aspect, the term "insulin related growth factor" refers to recombinant insulin related growth factor. In one aspect, the term "insulin related growth factor" refers to insulin related growth factor purified from a cell or tissue In one aspect the insulin related growth factor is from a vertebrate. In one aspect the insulin related growth factor is from a mammal. In a further embodiment the insulin related growth factor is human. In another aspect the recombinant insulin related growth factor is produced from a plant cell. In one particularly preferred embodiment the recombinant insulin related growth factor is produced from transgenic rice (Oryza sativa) Representative species and Gene bank accession numbers for various species of insulin related growth factor are listed below in **Table D5**.

Table D5
Exemplary insulin related growth factor genes

Species	Gene Bank Accession number
Homo sapiens	P01343 P05019 CAA01954.1 CAA40093.1 CAA40092.1
Bos taurus	NP_001071296
Mus musculus	NP_001104746.1 NP_034642.2 NP_908941.1 NP_001104745.1 NP_001104744.1
Xenopus laevis (African clawed frog)	Q90WW4
Danio rerio (zebrafish	NP_571900

The **insulin related growth factor** may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of

insulin related growth factor, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of **insulin related growth factor**. **Insulin related growth factor** which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native **insulin related growth factor** amino acid sequences, for example, to any of the native **insulin related growth factor** gene sequences listed in **Table D5**. Alternatively, the **insulin related growth factor** may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with **insulin related growth factor** listed in **Table D5**. In a preferred embodiment, the **insulin related growth factor** for use in any of the methods of the present invention is at least 80% identical to the mature human **insulin related growth factor**.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 5, about 1 to 10, about 1 to 15, about 1 to 20 or about 1 to 25. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 0.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 1 about 1 to 2, about 1 to 3, about 1 to 4 or about 1 to 5. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.5 to 0.8 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.5 about 1 to 0.75, about 1 to 1.0 about 1 to 1.25, about 1 to 1.5, about 1 to 1.75 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.8 to 1.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.25 about 1 to 0 33, about 1 to 0.66 about 1 to 1 00, about 1 to 1 33, about 1 to 1 66 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 1.0 to about 1 5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.8, about 1 to 1 or about 1 to 1.2. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 1.5 g/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 20, about 1 to 100. or about 1 to 200. In one aspect of any of these supplements the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 500, about 1 to 1000, or about 1 to 2000. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 3000. about 1 to 4000, or about 1 to 5000.

In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and albumin mixed in a ratio of transferrin to albumin of about 1 to 80, about 1 to 100 or about 1 to 120. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 3 to about 8 u g/L. In one aspect the supplement exhibits at least an 80% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of IGF-1 and recombinant albumin mixed in a ratio of IGF-1 to albumin of about 1 to 100000, about 1 to 10000, or about 1 to 1000 In one aspect of any of these supplements, the concentration of IGF-1 in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In another aspect of the invention, the supplements contain one or more additional factors selected from the group consisting of a growth factor, sodium selenate and ethanolamine

In another aspect of the invention, the growth factors are independently selected from insulin, Epidermal Growth Factor (EGF), Fibroblast Growth Factors 1-23 (FGF), Insulin-like Growth Factor-1(IGF), keratinocyte growth factors 1 & 2(KGF), and Leukemia Inhibitory Factor (LIF). In one aspect the growth factor is insulin. In another aspect the growth factor is transferrin.

In one aspect, the supplements of the invention may be prepared by mixing the isolated cell culture components in aqueous solution.

Liquids of known concentration can also be combined containing one component part A (albumin or another cell culture component), to a liquid containing part B (such as a transferrin related protein or IGF-1) to obtain a ratio that contains the desired ratios of the components

Powdered, lyophilized, or otherwise dried powder (albumin) can be added directly to an aqueous solution containing the cell culture component (such as transferrin related protein) in order to obtain a ratio based on dry weight of albumin at any desired range with respect to the other cell culture components Powdered, lyophilized, or otherwise dried albumin can also be blended with the cell culture component powder on a mass to mass basis to obtain a ratio that is completely based on gravimetrics. The resulting powder can be dissolved at concentrations ranging from very low (picomolar) to very high concentrations (millimolar) in suitable buffers that are common to the art to reconstitute the cell culture components

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin and one or more transferrin related proteins and /or IGF-1. Such supplements will commonly be prepared as sterile liquid or powder form The total amount of transferrin related protein in the composition may vary from 50% to about 0.0001% of weight of the cell culture component. In other aspects the amount of transferrin related protein in the composition may vary from about 0.01% to about 0.02%, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0 02% to about 0.06%, or about 0.02% to about 0.08%. In another aspect the amount of transferrin related protein in the composition is greater than about 0 01%, or more preferably greater than about 0.05%, or more preferably greater than about 0.1% wt /wt, or more preferably greater than about 0.2% wt/wt transferrin related protein with respect to albumin.

In one aspect of any of the claimed supplements, the recombinant albumin is essentially free of endotoxin and detergents. In another aspect the albumin has less than about 1 EU /mg of endotoxin. In yet another aspect, the albumin contains less than about 10 ppm detergent. In another aspect of any of the claimed supplements, the albumin has a purity of greater than 95 %.

In another of any of the claimed supplements, the supplement comprises recombinant albumin which is bound to a rice heat shock protein, wherein the complex has less than about 1 EU of endotoxin and is at least 95 % pure with respect to the albumin. In one aspect the recombinant albumin is produced in rice.

In another aspect of any of these methods the supplement contains recombinant albumin as the cell culture component, and the albumin is essentially free of aggregated albumin In another aspect of any of these supplements the recombinant albumin has less than about 2 % aggregated albumin.

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin with one or more bound hsps. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of hsp in the albumin composition may vary from 5 % to about 0.001% of weight of albumin. In other aspects the amount of hsp in the albumin composition may vary from about 0.01% to about 0.02 %, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08 %. In another aspect the amount of hsp in the albumin composition is greater than about 0 02%, or more preferably greater than about 0.03%, or more preferably greater than about 0.04% wt /wt, or more preferably greater than about 0.05% wt/wt hsp with respect to albumin

III. Production of Cell culture media components

Albumin and transferrin related proteins, as well as other cell culture media components for use in the supplements of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (see below), or by chemical synthesis, using, for instance, automated peptide synthesizers, or any combination of such methods. The means for preparing such polypeptides are well understood in the art.

For recombinant production, host cells can be genetically engineered to incorporate nucleic acids encoding the cell culture media component. Typically the nucleic acid will be codon optimized for high level expression in the expression system of choice, and incorporated into an expression vector to enable the expression of the protein of interest in the host cell. Vectors can exist as circular, double stranded DNA, and range in size form a few kilobases (kb) to hundreds of kb Preferred cloning vectors have been modified from naturally occurring plasmids to facilitate the cloning and recombinant manipulation of polynucleotide sequences. Many such vectors are well known in the art and commercially available; see for example, by Sambrook (In. "Molecular Cloning: A Laboratory Manual," second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)), Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608(1980).

General and specific techniques for producing proteins from plant cells may be obtained from the following patents and applications, each of which is incorporated herein in its entirety by reference: U.S. Pat Pub. No. 2003/0172403 A1 ("Plant Transcription Factors and Enhanced Gene Expression"); U.S. Pat. No. 6,991,824 ("Expression of Human Milk Proteins in Transgenic

Plants"); U.S. Pat. Pub. No. 2003/0221223 ("Human Blood Proteins Expressed in Monocot Seeds"); U.S. Pat. Pub. No. 2004-0078851 ("Production of Human Growth Factors in Monocot Seeds"); U S. Pat. Pub. No. 2004/0063617 ("Method of Making an Anti-infective Composition for Treating Oral Infections"), and international application no. PCT/US2004/041083 ("High- level Expression of Fusion Polypeptides in Plant Seeds Utilizing Seed- Storage Proteins as Fusion Carriers"). Other general and specific techniques for producing proteins from plant cells may be obtained, for example, from the following references, each of which is incorporated herein in its entirety by reference: U.S. Patent No. 5,693,507, U.S. Patent No. 5,932,479, U.S Patent No. 6,642,053, and 6,680,426 (each titled "Genetic Engineering of Plant Chloroplasts"); U S. Pat. Pub. No. 2005/0066384 ("Site-Targeted Transformation Using Amplification Vectors"); U.S. Pat. Pub. No. 2005/0221323 ("Amplification Vectors Based on Trans-Splicing"); U.S. Pat. Pub. No. 2006/0026718 ("Method of Controlling Cellular Processes in Plants"); and U.S. Pat. Pub. No. 2006/0075524 (Method of Controlling A Cellular Process in a Multi- Cellular Organism"); Marillonnet et at., Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants, Nature Biotech. (2005) 23(6): 718-723.

Expression vectors include plasmids, episomes, cosmids retroviruses or phages; the expression vector can be used to express a DNA sequence encoding the cell culture media component, and in one aspect comprises an assembly of expression control sequences. The choice of promoter and other regulatory elements can vary according to the intended host cell, and many such elements are available commercially, and can be readily assembled from isolated components such as the Gateway system from Invitrogen, (CA, USA). Expression systems for cell culture media components can be stable or transient expression systems.

Representative commercially available viral expression vectors include, but are not limited to, the adenovirus-based systems, such as the Per.C6 system available from Crucell, Inc., lentiviral-based systems such as pLP1 from Invitrogen, and retroviral vectors such as tobacco mosaic virus based vectors (Lindbo et al., BMC Biotechnol. (2007) 7 52-58)

An episomal expression vector is able to replicate in the host cell, and persists as an extrachromosomal episome within the host cell in the presence of appropriate selective pressure. (See for example. Conese et al., Gene Therapy 11: 1735-1742 (2004)). Representative commercially available episomal expression vectors include, but are not limited to, episomal plasmids that utilize Epstein Barr Nuclear Antigen 1 (EBNA1) and the Epstein Barr Virus (EBV) origin of replication (oriP), specific examples include the vectors pREP4, pCEP4, pREP7 from Invitrogen. The host range of EBV based vectors can be increased to virtually any

eukaryotic cell type through the co-expression of EBNA1 binding protein 2 (EPB2) (Kapoor et al., EMBO. J. 20: 222-230 (2001)), vectors pcDNA3.1 from Invitrogen, and pBK-CMV from Stratagene represent non-limiting examples of an episomal vector that uses T-antigen and the SV40 origin of replication in lieu of EBNA1 and oriP.

An integrating expression vector can randomly integrate into the host cell's DNA, or can include a recombination site to enable the specific recombination between the expression vector and the host cells chromosome. Such integrating expression vectors can utilize the endogenous expression control sequences of the host cell's chromosomes to effect expression of the desired protein. Examples of vectors that integrate in a site specific manner include, for example, components of the flp-in system from Invitrogen (e.g., pcDNA™5/FRT), or the cre-lox system, such as can be found in the pExchange-6 Core Vectors from Stratagene. Examples of vectors that integrate into host cell chromosomes in a random fashion include, for example, pcDNA3.1 (when introduced in the absence of T-antigen) from Invitrogen, pCI or pFN10A (ACT) Flexi® from Promega.

Alternatively, the expression vector can be used to introduce and integrate a strong promoter or enhancer sequences into a locus in the cell so as to modulate the expression of an endogenous gene of interest (Capecchi MR. Nat Rev Genet. (2005); 6 (6):507-12; Schindehutte et al., Stem Cells (2005); 23 (1):10-5). This approach can also be used to insert an inducible promoter, such as the Tet-On promoter (US Patents 5,464,758 and 5,814,618), in to the genomic DNA of the cell so as to provide inducible expression of an endogenous gene of interest. The activating construct can also include targeting sequence(s) to enable homologous or non-homologous recombination of the activating sequence into a desired locus specific for the gene of interest (see for example, Garcia-Otin & Guillou. Front Biosci. (2006) 11:1108-36). Alternatively, an inducible recombinase system, such as the Cre-ER system, can be used to activate a transgene in the presence of 4-hydroxytamoxifen (Indra et al. Nuc. Acid. Res. (1999) 27 (22). 4324-4327; Nuc. Acid. Res. (2000) 28(23). e99; and U.S. Patent No. 7,112,715).

Suitable cells for producing the cell culture media components include prokaryotic cells, yeasts, insect cells, plant expression systems and mammalian expression systems. Within these general guidelines, useful microbial hosts include, but are not limited to, bacteria from the genera Bacillus, Escherichia (such as *E. coli*), Pseudomonas, Streptomyces, Salmonella, Erwinia, *Bacillus subtilis*, *Bacillus brevis*, the various strains of *Escherichia coli* (e.g., HB101, (ATCC NO. 33694) DH5α, DH10 and MC1061 (ATCC NO. 53338)).

Many strains of yeast are known to those skilled in the art and are also available as host cells for the expression of cell culture media components including those from the genera Hansenula, Kluyveromyces, Pichia, Rhino-sporidium, Saccharomyces, and Schizosaccharomyces, and other fungi. Preferred yeast cells include, for example, *Saccharomyces cerivisae* and *Pichia pastoris*.

Additionally, where desired, insect cell systems can be utilized to produce the cell culture media components for use in the supplements and methods of the present invention. Such systems are described, for example, by Kitts et al., Biotechniques, 14:810-817 (1993); Lucklow, Curr. Opin Biotechnol., 4:564-572 (1993); and Lucklow et al (J. Virol., 67:4566-4579 (1993). Preferred insect cells include Sf-9 and Hi5 (Invitrogen, Carlsbad, Calif).

Many suitable plant expression systems can be used for the expression of cell culture media components examples includes for example, any monocot or dicot plant. Suitable monocot plants include without limitation, rice, barley, wheat, rye, corn, millet, triticale, or sorghum, preferably rice. Other suitable plants include Arabidopsis, Alfalfa, tobacco, peanut and soybean.

In general, expression vectors for use in plants include the following operably linked elements that constitute a chimeric gene: a promoter derived from a gene encoding a plant protein, operatively linked to a gene encoding a cell culture media component. The promoter region is chosen to be regulated in a manner allowing for induction under seed-maturation conditions. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, corn, millet, triticale or sorghum.

In one aspect, the chimeric gene includes a promoter which exhibits increased expression during seed maturation. Examples of such promoters include the maturation-specific promoter region associated with one of the following maturation-specific monocot plant storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

In one aspect, the chimeric gene includes i) a promoter from the gene of a maturation-specific monocot plant storage protein, ii) a first DNA sequence, operably linked to the promoter, encoding a monocot plant seed-specific signal sequence (such as an N-terminal leader sequence or a C-terminal trailer sequence) capable of targeting a polypeptide linked thereto to an endosperm cell, preferably an endosperm-cell organelle, such as a protein storage body, and iii) a second DNA sequence, linked in translation frame with the first DNA sequence, encoding a

cell culture media component. The signal sequence is preferably cleaved from the cell culture media component in the plant cell.

The chimeric gene, in turn, is typically placed in a suitable plant-transformation vector having (i) companion sequences upstream and/or downstream of the chimeric gene which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the desired plant host; (ii) a selectable marker sequence; and (iii) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells.

At least one selective purification tag and/or at least one specific protease cleavage site may be provided for eventual release of the cell culture media component from the monocot seed storage protein carrier. For example, a strategic methionine or tryptophan residue providing a chemical cleavage site may be engineered in frame between the domains for release of the cell culture media component from the endogenous plant protein Other selective protease cleavage sites include, but are not limited to enterokinase (ek), Factor Xa, thrombin, V8 protease, GENENASE™(a variant of subtilisin BPN'), α-lytic protease or tobacco etch virus protease. Alternatively, cleavage of the fusion protein could be performed via chemical cleaving agents such as cyanogen bromide or N-chlorosuccinimide.

A number of suitable mammalian host cells are also known in the art and many are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209. Examples include, but are not limited to, mammalian cells, such as Chinese hamster ovary cells (CHO) (ATCC No. CCL61) CHO DHFR-cells (Urlaub et al., Proc. Natl. Acad Sci. USA, 97:4216-4220 (1980)), human embryonic kidney (HEK) 293 or 293T cells (ATCC No. CRL1573), or 3T3 cells (ATCC No. CCL92). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 (ATCC No. CRL1650) and COS-7 cell lines (ATCC No. CRL1651), and the CV-1 cell line (ATCC No. CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines.

Cell-free transcription and translation systems can also be employed to produce such proteins using the DNA constructs (or RNAs derived from the DNA constructs) of the present invention.

Production of recombinant proteins of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides encoding the cell culture media component and to host cells which are genetically engineered with such expression systems and to the production of such proteins by recombinant techniques. In one embodiment the host cell endogenously expresses a heat shock protein of interest.

In cases where purification of the expressed proteins of the supplement of the invention are necessary, proteins of the present invention can be recovered from either the cellular environment, before lysing the cells, or after cell lysis The proteins can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. High performance liquid chromatography is also employed for purification.

A search of patents, published patent applications, and related publications will provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying albumin. For example, US Patent Nos. 4,075,197; 4,086,222; 4,093,612; 4,097,473; 4,136,094; 4,228,154; 5,250,662; 5,656,729; 5,677,424; 5,710,253; 5,728,553; 5,994,507; 6,001,974; 6,638,740; 6,617,133 and 7,423,124 disclose various processes for purifying albumin. In one aspect, the albumin for use in the present invention is purified using any of these art recognized processes listed above, and then mixed in aqueous solution with a heat shock protein Methods for the purification of heat shock proteins, including anion exchange chromatography and ATP agarose affinity chromatography are well known in the art. (Welch & Feramisco, J. Biol. Chem. 257 (24)14949-14959; (1982); Welch & Feramisco, Mol. Cell. Biol. 5 (6) 1229-1237 (1985) Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification

In one preferred aspect recombinant albumin is purified using procedures that enable the direct co-purification of both recombinant albumin and a heat shock protein, or hsp protein complex, for example as described below and in the Examples section In one aspect the recombinant albumin is produced in rice, and the heat shock protein is an endogenous rice heat shock protein.

In one aspect, of this method, expression vectors are used to increase the expression of the recombinant albumin in the host cell, while the expression of the host cells endogenous heat shock proteins is accomplished by activating the expression of the host cells hsp genes. In another aspect expression vectors are used to increase the expression of the heat shock protein. In another aspect expression vectors are used to increase the expression of the heat shock protein and albumin. In another aspect the nucleic acid sequence encoding a heat shock protein and albumin are located in the same expression vector

Due to the similar electronegativity of albumin and hsp70, anion exchange chromatography is the preferred method to prepare albumin enriched in Hsps. For example, both albumin and Hsp70 bind to anion exchange columns with resins consisting of either quaternary amine or diethylaminoethyl mounted on a bead that is suitable for the ion exchange of polypeptides (large molecular exclusion limit and of suitable size) at high pH (7.5 and above). Examples of such resins are General Electric (GE) Q Sepharose and GE DEAE Sepharose. Due to their similar electronegativity, utilizing low pH conditions (below pH 6.5) allows for the co-purification of the two molecules on cation exchangers as well. Examples of such cation exchangers are GE Carboxymethyl Sepharose and Sulfonic acid Sepharose based resins. Because the albumin and Hsp70 have similar isoelectric points, mixed mode resins may also be employed for the co-purification of albumin and Hsp70. Since both Hsp70 and Albumin are well known to bind to fatty acids and other hydrophobic molecules, it is also possible to co-purify albumin and Hsp70 on a hydrophobic based resin such as octyl sepharose (GE). Due the similar size of Hsp70 proteins and Albumin (65-75 kDa), co-purification of the two proteins and enrichment of Hsp70 by tangential flow ultrafiltration utilizing both higher and lower molecular exclusions than 65-75 kDa may also be employed to co-purify and thus enrich Albumin with hsps.

Also due to their similar molecular weights, any method that separates polypeptides based on size should effectively co-purify albumin and hsp70 such as molecular sieves and gel filtration or size exclusion chromatography. In addition, due to the similar nature of Hsp70 and Albumin in terms of hydrophobicity and electronegativity or surface charge may be co-purified by precipitation under a number of conditions. Some of those conditions are precipitation by ammonium sulfate, precipitation by denaturants such as urea, or precipitation based on isoelectric point and solubility

The methods are also applicable to enrich albumin with hsps from other sources. For example albumin derived from native and transgenic animal feedstock serum, as well as albumin

produced from recombinant organisms and tissue culture systems based on prokaryotic and eukaryotic cells, including, vertebrate cells such as mammalian cells, and non vertebrate cells, such as insects, as well as plant, and fungi such as yeast, and the like

A search of patents, published patent applications, and related publications will also provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying transferrin related proteins. For example, US Patent Nos 7,368,141; 7,354,902; 5,708,149. 5,596,082; 5,169,936; 4,791,193; and 4,667,018 disclose various processes for purifying transferrin related proteins

IV Exemplary Cells

Without wishing to be bound by theory, it is contemplated that any cell which is susceptible to apoptosis may be used in the methods of the invention, including primary cells, immortalized cells, differentiated cells, undifferentiated cells or cells, such as stem cells, with varying degrees of specialization. In a particular embodiment, cells used in the methods of the invention are transfected with a nucleic acid molecule comprising a nucleotide sequence encoding a protein of interest, e.g., a therapeutic protein or an antibody.

In a particular embodiment, the cells used in the methods of the invention are eukaryotic cells, e.g., mammalian cells. Examples of mammalian cells include, but are not limited to, for example, human B-cells, and T cells, and derivatives thereof, such as hybridomas, and cell expressing markers of B or T cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J Gen Virol., 36:59 (1977)), baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); CHO-K1 cell (ATCC CCL-61), human PER.C6 cells (Crucell, NV), mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci., 383:44-68 (1982)). MRC 5 cells; FS4 cells; NSO mouse myeloma cells (ECACC; SIGMA), and a human hepatoma line (Hep G2) Additional examples of useful cell lines include, but are not limited to, HT1080 cells (ATCC CCL 121), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB

carcinoma cells (ATCC CCL 17), 2780AD ovarian carcinoma cells (see Van der Blick, A M. et al , Cancer Res. 48:5927-5932 (1988), Raji cells (ATCC CCL 86), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells (ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607), WI-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. These and other cells and cell lines are available commercially, for example from the American Type Culture Collection (Virginia, USA) Many other cell lines are known in the art and will be familiar to the ordinarily skilled artisan; such cell lines therefore can be used equally well in the methods of the present invention. In a particular embodiment, cells used in the methods of the invention are CHO cells or NSO cells. Hybridomas and antibody-producing cells may also be used in the methods of the invention

In another embodiment, cells used in any of the methods of the invention are stem cells. Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate in vitro into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts

Types of human embryonic stem cells that may be used in any of the methods of the invention include established lines of human embryonic cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga). In one embodiment, Human embryonic stem cells are prepared as described by Thomson et al. (U.S. Pat. No. 5,843,780: Science 282:1145, 1998; Curr Top Dev Biol. 38:133 ff., 1998; Proc. Natl Acad. Sci. U.S.A. 92:7844, 1995)

Additionally, hybridoma cells can also be used in the methods of the invention. The term "hybridoma" refers to a hybrid cell line produced by the fusion of an immortal cell line of immunologic origin and an antibody producing cell. The term encompasses progeny of heterohybrid myeloma fusions, which are the result of a fusion with human cells and a murine myeloma cell line subsequently fused with a plasma cell, commonly known as a trioma cell line. Furthermore, the term is meant to include any immortalized hybrid cell line which produces antibodies such as, for example, quadromas See. e.g., Milstein et al., Nature. 537:3053 (1983) The hybrid cell lines can be of any species, including human, rabbit and mouse.

In some embodiments, a cell line used in the methods of the invention is an antibody-producing cell line. Antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan See, e g., Current Protocols in Immunology, Coligan et al., Eds , Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements. In general, any cell suitable for recombinant protein expression in cell culture can be used in the methods of the invention.

In some embodiments, the cells used in the methods of the present invention may include a heterologous nucleic acid molecule which encodes a desired recombinant protein, e.g., a therapeutic protein or antibody which is desired to be produced using the methods of the invention. In a particular embodiment, the methods of the present invention are useful for producing high titers of a desired recombinant protein, e.g., a therapeutic protein or antibody, in the presence of reduced levels of one or more contaminants.

V. Cell Culture Media

Any suitable culture medium or feed medium suitable for cell growth and protein production may be used in the methods of the invention. Suitable culture or feed mediums are chosen for their compatibility with the host cells and process of interest. Suitable culture or feed mediums are well known in the art and include, but are not limited to, commercial media such as Ham's F10 (SIGMA), Minimal Essential Medium (SIGMA), RPMI-1640 (SIGMA), and Dulbecco's Modified Eagle's Medium SIGMA) are suitable for culturing the animal cells. In addition, any of the media described in Ham and Wallace, (1979) Meth. Enz . 58:44; Barnes and Sato,(1980) Anal Biochem., 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469 or 4,560,655; International Publication Nos. WO 90/03430; and WO 87/00195 may be used.

Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The necessary growth factors for a particular cell are readily determined empirically without undue experimentation, as described for example in Mammalian Cell Culture (Mather, J. P ed., Plenum Press, N.Y (1984), and Barnes and Sato, Cell, 22:649 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the protein of interest in recombinant vertebrate cell culture are described in Gething et al , Nature, 293.620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117.058. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

VI. Exemplary Cell Culture Expression Products

In one aspect of any of the claimed methods, the supplements of the invention are used to improve the viability and growth of a cell which is used to express and produce a protein of interest. The cell may express the protein of interest endogenously or may be an engineered cell line that has been modified genetically to express the protein of interest at levels above background for that cell.

Cells may be genetically modified to express a protein by transformation with a nucleic acid encoding the protein of interest, or by transformation of an activating sequence that promotes the expression of an endogenous gene. In one aspect the protein of interest may be expressed from an expression vector, in which a coding sequence for the protein of interest is operably linked to an expression control sequences, to enable either constitutive or inducible expression, as is known in the art

The protein of interest may be any protein, or fragment thereof, which is of commercial, therapeutic or diagnostic value including without limitation cytokines, chemokines, hormones, antibodies, anti-oxidant molecules, engineered immunoglobulin-like molecules, a single chain antibodies, a humanized antibodies, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, transdominant negative mutants of a target protein, toxins, conditional toxins, antigens, a tumor suppresser proteins, growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). The protein of interest may also comprise pro-drug activating enzymes.

In some embodiments, the protein of interest comprises a glycoprotein, or any other protein which has one or more post-translational modifications. For example, any protein which is suitable for production in a eukaryotic host may be expressed using the methods and compositions described here.

The methods of the invention can be used to produce any desired recombinant protein or fragment thereof. In some embodiments, a recombinant protein produced using the methods described herein is a therapeutic protein. In other embodiments, the recombinant protein is an antibody or functional fragment thereof. Antibodies which may be produced using the methods of the invention include, for example, polyclonal, monoclonal, monospecific, polyspecific, fully human, humanized, single-chain, chimeric, hybrid, CDR grafted,. It may comprise a full length IgG1 antibody or an antigen-binding fragments thereof, such as, for example, Fab, $F(ab')_2$, Fv, and scfv.

Antibodies within the scope of the present invention include, but are not limited to, anti-HER2 antibodies including Trastuzumab (HERCEPTIN™) (Carter et al. Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN™), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108, B1, or Tositumomab (BEXXAR™.); anti-IL-8 (St John et al., Chest, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN™ (Kim et al., Growth Factors, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., Transplant Intl. 4:3-7 (1991), and Hourmant et al., Transplantation

58:377-380 (1994)); anti-IgE (Presta et al., J. Immunol. 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No PCT/US98/13410 filed Jun 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-alpha, antibodies including cA2 (REMICADE™), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. J. Immunol. 156(4).1646-1653 (1996), and Dhainaut et al Crit. Care Med. 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human alpha 4 beta 7 integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S Pat No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT™) and (ZENAPAX™) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al Arthritis Rheum 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. Nature 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fc gamma RI as in Graziano et al. J. Immunol. 155(10):4996-5002 (1995), anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. Cancer Res. 55(23Suppl): 5935s-5945s (1995), antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. Cancer Res. 55(23). 5852s-5856s (1995); and Richman et al. Cancer Res. 55(23 Supp). 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. Eur J. Immunol. 26(1):1-9 (1996)), anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. J. Immunol. 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. Cancer Res 55(23 Suppl).5908s-5910s (1995) and CMA-676 or CDP771, anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. Cancer Res 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX™.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO™ anti-RSV antibodies such as MEDI-493 (SYNAGIS™.); anti-CMV antibodies such as PROTOVIR™.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR™; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-.alpha.v.beta.3 antibody VITAXIN™; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33), anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a,

and CD40.

The recombinant protein may be a cellular protein such as a receptor (e.g., membrane bound or cytosolic) or a structural protein (e.g. a cytoskeleton protein). The recombinant protein may be cellular factor secreted by the cell or used internally in one or more signal transduction pathways. Non limiting examples include, but are not limited to, CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1, IL-2, IL-3, IL-7, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF, EGF receptor, VEGF receptor, hepatocyte growth factor, osteoprotegerin ligand, interferon gamma, B lymphocyte stimulator C5 complement TAG-72, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2, and CTLA4 (which is a cytotoxic T lymphocyte-associated antigen).

The recombinant protein may also be derived from an infectious agent such as a virus, a bacteria, or fungus. The protein may be derived from a bacterial membrane or cell wall, or may be derived from the bacterial cytosol. The protein may be a bacterial or yeast enzyme, transcription factor, or structural protein. The bacterial or yeast protein may be membrane bound, cytsolic, or secreted. Examples of infectious agents include, but are not limited to, Streptococcus mutans, and Staphlycoccus aureus, and Candida albicans.

The methods of the invention can also be used to produce recombinant fusion proteins comprising all or part of any of the above-mentioned proteins. For example, recombinant fusion proteins comprising one of the above-mentioned proteins plus a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an antibody, or a substantially similar protein, can be produced using the methods of the invention. See e.g. International Application No. WO 94/10308; Lovejoy et al. (1993), Science 259:1288-1293; Harbury et al. (1993), Science 262: 1401-05; Harbury et al. (1994), Nature 371:80-83; Hang.kansson et al. (1999), Structure 7:255-64.

Also encompassed by this invention are pharmaceutical compositions including one or more recombinant proteins produced by the methods described herein. In some embodiments, pharmaceutical compositions further include a pharmaceutically acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or

liquid filler, diluents or encapsulating substances which are suitable for administration into a subject.

VII Stem Cells

In one embodiment, human embryonic stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation, comprising a supplement of the invention The growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type and further comprising a supplement of the present invention. Alternatively, the growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a chemically defined medium comprising a supplement of the present invention. Examples of feeder-free, serum free culture systems in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal include those disclosed in US patent applications, US20050148070, US20050244962, US20050233446, U.S. Pat. No.6,800,480, and PCT publications WO2005065354 and WO2005086845.

In an alternate embodiment, human embryonic stem cells are initially cultured with a layer of feeder cells that support the human embryonic stem cells and further comprising a supplement of the present invention The human embryonic are then transferred to a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation and which further comprises a supplement of the present invention In any of these approaches, the use of the supplements of the invention results in significantly enhanced rates of cell growth and improved cell viability.

Examples of conditioned media suitable for use with the supplements of the present invention are disclosed in US20020072117, U.S. Pat. No. 6,642,048, WO2005014799, and Xu et al (Stem Cells 22: 972-980, 2004) An example of a chemically defined medium suitable for use with the supplements of the present invention may be found in US20070010011.

Examples of feeder cells include feeder cells selected from the group consisting of a fibroblast cell, a MRC-5 cell, an embryonic kidney cell, a mesenchymal cell, an osteosarcoma cell, a keratinocyte, a chondrocyte, a Fallopian ductal epithelial cell, a liver cell, a cardiac cell, a bone marrow stromal cell, a granulosa cell, a skeletal muscle cell, a muscle cell and an aortic

endothelial cell. In a preferred embodiment, the MRC-5 cell, has ATCC Catalog Number 55-X; the transformed and has ATCC Accession Number CRL-2309; the human osteosarcoma cell has ATCC Accession Number HTB-96; and the mesenchymal cell is a human fetal palatal mesenchymal cell with ATCC Accession Number CRL-1486. In other preferred embodiments the human fibroblast cell is a skin keloid fibroblast, KEL FIB and has ATCC Accession Number CRL-1762, or is a fetal skin fibroblast cell; and the bone marrow stromal cell, HS-5, has ATCC Accession Number CRL-11882.

Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco #15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522, human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

In one embodiment, the human embryonic stem cells are plated onto a suitable culture substrate that is treated prior to treatment according to the methods of the present invention, with a composition comprising a supplement of the present invention. In one embodiment, the treatment is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL (Becton Dickenson). MATRIGEL is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

Other extracellular matrix components and component mixtures are suitable as an alternative and can be used with the supplements of the present invention. This may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations with a supplement of the present invention.

In another embodiment, the invention encompasses an embryonic stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention. In one embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention

In another embodiment the invention encompasses an embryonic stem cell culture, comprising a human embryonic stem cell and a human feeder cell culture comprising a supplement of the

invention. In another embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a human feeder cell culture comprising a supplement of the invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing pluripotency markers, comprising the steps of:

Culturing human embryonic stem cells,

Differentiating the human embryonic stem cells into cells expressing pluripotency markers, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing markers, characteristic of ectodermal, endodermal or mesodermal cells, comprising the steps of:

Culturing pluripotency stem cells;

Differentiating the pluripotency stem cells into cells expressing markers characteristic of ectodermal, endodermal or mesodermal cells, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In any of these methods, the stem cells can be differentiated into cells expressing markers characteristic of an endodermal, ectodermal or mesodermal lineage by any method in the art. For example, cells expressing pluripotency markers may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 23, 1534-1541 (2005), by Shinozaki et al, Development 131, 1651-1662 (2004), McLean et al., Stem Cells 25, 29-38 (2007), D'Amour et al., Nature Biotechnology 24, 1392-1401 (2006).

Cells expressing markers characteristic of the endoderm lineage may be further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art. For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 24, 1392-1401 (2006), wherein the differentiation is conducted in the presence of a supplement of the present invention.

In one aspect of any of these methods of differentiation, the human embryonic stem cells are

cultured and differentiated on a tissue culture substrate coated with an extracellular matrix. The extracellular matrix may be a solubilized basement membrane preparation extracted from mouse sarcoma cells (which is sold by BD Biosciences under the trade name MATRIGEL). Alternatively, the extracellular matrix may be growth factor-reduced MATRIGEL. Alternatively, the extracellular matrix may be fibronectin. In an alternate embodiment, the human embryonic stem cells are cultured and differentiated on tissue culture substrate coated with human serum. In one aspect, the tissue culture substrate is coated with extracellular matrix and a supplement of the present invention.

The extracellular matrix may be diluted prior to coating the tissue culture substrate. Examples of suitable methods for diluting the extracellular matrix and for coating the tissue culture substrate may be found in Kleinman, H. K., et al , Biochemistry 25:312 (1986), and Hadley, M A., et al., J. Cell. Biol 101:1511 (1985).

In one aspect of the methods of stem cell differentiation, the culture medium should contain sufficiently low concentrations of certain factors to allow the differentiation of human embryonic stem cells to cells of endoderm, ectoderm or mesoderm lineage. such as, for example insulin and IGF (as disclosed in WO2006020919). This may be achieved by lowering the serum concentration, or alternatively, by using chemically defined media that lacks insulin and IGF. Examples of chemically defined media are disclosed in Wiles et al (Exp Cell Res. 1999 Feb. 25, 247(1): 241-8.). In a preferred embodiment, of any of these methods. the culture media comprises a supplement of the present invention.

The culture medium may also contain at least one other additional factor that may enhance the formation of cells expressing markers characteristic of endoderm, mesoderm or ectoderm lineage from human embryonic stem cells. The at least one additional factor may be, for example, nicotinamide, members of TGF-β family, including TGF-β1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (GDF-5, -6, -8, -10, 11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS, noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine 1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA),

keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof. In a preferred embodiment, of any of these methods, the culture media containing at least one additional factor listed above, further comprises a supplement of the present invention.

The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No: HTB-79), BxPC-3 (ATCC No. CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241). In a preferred embodiment, of any of these methods, the conditioned media further comprises a supplement of the present invention.

In another embodiment, the invention encompasses a method of using the cell or tissue of any of the aforementioned stem cells for the experimental, therapeutic and prophylactic treatment of a disease or condition in a human or animal. Preferably, the disease is selected from the group consisting of Parkinson's, Alzheimer's, Multiple Sclerosis, spinal cord injuries, stroke, macular degeneration, burns, liver failure, heart disease, diabetes, Duchenne's muscular dystrophy, osteogenesis imperfecta, osteoarthritis, rheumatoid arthritis, anemia, leukemia, breast cancer, solid tumors, and AIDS. In a preferred embodiment, the disease is Parkinson's or Alzheimer's. In a more preferred embodiment, the disease is Parkinson's.

VIII. Large Scale production of recombinant proteins

In one embodiment, the supplements of the present invention can be used to produce a protein of interest by growing host cells in the presence of the supplement. In one embodiment, the cell culture is performed in a stirred tank bioreactor system and a fed batch culture procedure is employed. In another embodiment a wave disposable bioreactor is employed. In the bioreactor system, the size of the bioreactors are sufficiently large to produce the desired amount of protein of interest, such as 1,000 Liter or 12,000 Liter sizes, but are not limited to such sizes as much smaller (i.e., 2 Liter, 400 Liter) or larger (i.e., 25,000 Liter, 50,000 Liter) bioreactor vessels may be appropriate. In the preferred fed batch culture, the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells

and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process but at the termination of the culture process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cultured cells may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single step or multiple step culture procedure. In a single step culture, the host cells are inoculated into a culture environment and the method steps of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture, cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium comprising a supplement of the present invention suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase, cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 38° C. and preferably about 37° C. and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above, the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell culture environment during the production phase of the cell culture is controlled. According to the steps of the presently disclosed methods, the

addition of the supplements of the invention can be coordinated such that the desired content and quality of the protein of interest is achieved and maintained in the resulting cell culture fluid. In a preferred aspect, the production phase of the cell culture is preceded by a transition phase of the cell culture in which the addition of the supplements of the invention initiates the production phase of the cell culture.

In any of the above-described methods, it is contemplated that it may be desirable to include a desired amount of agent like butyrate or Trichostatin A in the cell culture medium in combination with a supplement of the invention. Various forms of butyrate and its salts are known in the art, such as butyric acid and sodium butyrate, and are publicly available from sources such as Sigma Chemical Co. Butyrate has been reported in the literature to enhance the productivity and protein expression of cell cultures [Arts et al , Biochem J., 310:171-176 (1995), Gorman et al , Nucleic Acids Res., 11.7631-7648 (1983); Krugh, Mol. Cell Biochem. 42:65-82 (1982); Lamotte et al., Cytotechnology, 29:55-64 (1999); Chotigeat et al., Cytotechnology, 15:217-221 (1994)]. Trichostatin A (TSA) is an inhibitor of histone deacetylase and may act similarly to butyrate in enhancing the productivity and protein expression in cell cultures [Medina et al., Cancer Research, 57:3697-3707 (1997)]. Although butyrate has some positive effects on protein expression, it is also appreciated in the art that at certain concentrations, butyrate can induce apoptosis in the cultured cells and thereby decrease viability of the culture as well as viable cell density [Hague et al., Int. J. Cancer, 55:498-505 (1993); Calabresse et al , Biochim. Biophys. Res. Comm., 195:31-38 (1993), Fillipovich et al., Biochim Biophys. Res. Comm., 198:257-265 (1994), Medina et al., Cancer Research, 57:3697-3707 (1997)]. In the methods of the present invention, a desired amount of butyrate or TSA may be added to the cell culture at the onset of the production phase and more preferably, may be added to the cell culture after a temperature shift has been implemented Butyrate or TSA can be added in a desired amount determined empirically by those skilled in the art, but preferably, butyrate is added to the cell culture at a concentration of about 1 to about 25 mM, and more preferably, at a concentration of about 1 to about 6 mM.

Expression of the protein of interest may be measured in a sample directly, for example, by ELISA, conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a

wide variety of labels, such as radionucleotides, fluorophors or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal and may be prepared in any mammal. Many are commercially available.

The supplements claimed herein can also be used to increase transfection efficiency and viability of cells during transfection. Conditions and reagents used in various transfection techniques, such as Lipofectamine are relatively toxic to the cells, while electroporation can severely stress a cell. The use of higher concentrations of transfection reagents, and more extensive electroporation conditions is preferred to achieve higher transfection efficiencies. Thus the addition of the supplements of the invention prior, with, and after transfection can result in higher transfection efficiencies, and higher yields of recombinant proteins.

The supplements of the invention can be used to express proteins of interest which induce apoptosis, such as Apo-2 ligand / TRAIL or Fas ligand. The presence of the supplements of the invention may block such apoptotic activity and allow for improved expression of the protein of interest.

In addition, the methods can be used to increase the viability of cells undergoing freezing / storage/thawing procedures. During these procedures generally cells can lose viability. The presence of apoptosis inhibitors added to the cell culture media can provide for increased cell viability and aid in reducing or eliminating the variability in cell viabilities between aliquots or

vials of cells.

IX. Kits

Also encompassed by the present invention are kits for promoting the viability of cells. In one embodiment, a kit according to the present invention comprises: (a) one or more reagents or devices for transfection and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding using the transfection device, transfection agent and supplement.

In another embodiment a kit according to the present invention comprises: (a) one or more reagents or devices for freezing or thawing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for freezing or thawing cell lines and the use of the reagents.

In another embodiment a kit according to the present invention comprises: (a) one or more tissue culture products for culturing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for dilution cloning techniques and the use of the reagents in such approaches.

All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and indicated individually to be incorporated by reference in its entirety. The following examples illustrate but are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Expression of recombinant human lactoferrin.

A. An Expression Vector For Human Lactoferrin Expression In Transgenic Rice

Complete nucleotide sequence of human mammary gland lactoferrin was codon optimized and synthesized by Operon Technologies (CA, USA). Human milk lactoferrin gene (Genbank accession number: HSU07642) was re-synthesized with codons most frequently used in translation of rice seed proteins in order to obtain optimal level of expression (**FIG. 1A-FIG. 1D**).

Although numbers of codons changed accounted for 22 46% of the entire sequence, amino acid composition remains identical to native human lactoferrin. The plasmid containing the codon-optimized gene was called Lac-ger Lac-ger was digested with SmaI/XhoI and the fragment containing the lactoferrin gene was cloned into pAPI141 that was partially digested with NaeI and completely digested with XhoI. For expression of hLF in rice seeds, the codon-optimized gene was operably linked to the rice endosperm-specific glutelin (Gt1) promoter and NOS terminator. The resulting plasmid was designated pAPI164 (**FIG. 2**).

B. Production system

Rice variety Taipei 309 (Oryza sativa, Japonica) was selected as the production system for recombinant human lactoferrin and transgenic rice events were generated by the particle bombardment of embryogenic rice calli with the plasmid pAPI164 and a companion marker plasmid containing the hygromycin phosphotransferase gene as a selectable marker. Fully developed, fertile rice plants were obtained by this procedure

C. High Level Protein Expression of recombinant human lactoferrin in rice grain

Expression of recombinant human lactoferrin was under the control of the seed maturation-specific promoter Gt1. The high level expression of recombinant human lactoferrin is evident in **FIG. 3** as independent transgenic rice events were screened. Total soluble proteins from mature rice seed extracts were run on Laemli gels and stained with Coomassie blue to visualize the proteins. An ~80kD recombinant lactoferrin protein was obtained in all transgenic lines as indicated by the stained gel. Expression levels of recombinant human lactoferrin corresponded to 0.5% of seed weight. The stable expression of recombinant human lactoferrin was monitored for 10 generations. The expression level is maintained at approximately 0.5% of seed weight of brown rice (**FIG. 4**).

Example 2: Expression & purification of recombinant human transferrin.

Purification of recombinant human from rice grain.

The rice grain is dehusked and milled to an average of 100 mesh flour using standard food industry procedures. Hulls and the solid material remaining after the extraction described below are destroyed. Recombinant human transferrin is extracted from the flour with extraction buffer (20 mM Tris-Base, pH 7.5), with stirring, for 40 minutes at room temperature. Filter aid (Cellpure 300) is added at 20g/L to the extraction mixture before filtration through a filter press with 10 kDa regenerated cellulose.

The filter press filtrate was diafiltered and concentrated with a 30 KDa molecular weight cutoff cellulose membrane into10 mM Tris-Base pH. 8.5 to prepare for DEAE chromatography. The DEAE column was washed with (20mM Tris-Base, 40mM Sodium Chloride, pH 7 5) prior to loading the concentrate onto the column. After loading, the column was washed with 3 volumes of 10 mM Tris-Base, 0.5% (v/v) Triton X-114, pH 8.5 buffer followed by 7 volumes of 10 mM Tris-Base, pH 8.5 buffer. Transferrin was then eluded with 20mM Tris-Base, 40mM Sodium Chloride, pH 7.5.

Transfernn that was recovered from the DEAE column was diafiltered and concentrated with a 10 KDa molecular weight cut off cellulose membrane into 10 mM sodium phosphate, 25mM sodium chloride, pH 7 0 buffer. After diafiltration, recombinant transferrin was filtered though a 0.2 u filter prior to drying.

Example 3: Expression of recombinant human albumin in rice grain.

Protein sequences of human albumin (HSA) from various data bases were compared. The consensus sequence represented by accession number P02768 (Swiss-Prot) was used as a base for gene codon-optimization for suitable expression of human albumin in rice grain (**FIG. 5A-FIG. 5D**). Gene synthesis was carried out by Blue Heron (Seattle, WA) and the synthetic fragment was inserted into pUC based vector to create pUC-HSA. After confirmation of correct DNA and protein sequences, pUC-HSA was digested with MlyI and XhoI. The fragment containing codon-optimized HSA gene was inserted into pAPI405, which had been precut with NaeI and XhoI. Plasmid API405 was a derivative of pAPI141 which included Gt1 promoter, Gt1 signal sequence and a nos terminator.

Insertion of MlyI/XhoI fragment into pAPI405 resulted in pAPI504 (**FIG. 6**). Plasmid API504 was then cleaved with HindIII and EcoRI. The HindIII/EcoRI fragment containing the entire expression cassette, Gt1 promoter, Gt1 signal sequence, codon-optimized HSA gene and nos terminator, was cloned into pJH2600 predigested with the same enzyme resulting in pAPI508 (**FIG. 7**). Plasmid JH2600 was a shuttle vector between E coli and Agrobacterium. After DNA sequence verification, pAPI508 was moved into Agrobacterium AGL-1 for rice transformation. Plasmid API504 was also used via bombardment-based transformation following the procedure described previously. Upon transformation, transgenic plants were generated and were sent to greenhouse where transgenic R0 plants grew to maturity and set R1 seeds.

To monitor the expression of HSA in rice seeds. 10 R1 seeds from each R0 plant were

extracted using 10 mL of extraction buffer (50 mM Tris-HCl pH 8.0. 150 mM NaCl). The supernatant was collected and the expression level from rice extracts was monitored by an ELISA (Bethyl Laboratories, Montgomery, TX) The results showed that the HSA expression level in rice transgenic seeds ranges from 0.01 to 0.85% of brown flour weight (0.1 to 8.5 grams/kg flour). The results of the eight events with highest expression levels are shown in the following table (**Table** E1).

Table E1 Recombinant HSA expression level in top eight events

Line Number	Express Level (g/kg flour)
508-30	5.5
508-17	8.0
508-71	8.7
508-73	4.0
508-77	8.5
508-83	8.5
508-101	3 0
508-113	4.0

Example 4: Purification of recombinant human lactoferrin as cell culture media component.

To prepare cell culture media supplemented with recombinant human lactoferrin, recombinant human lactoferrin was purified from rice flour. Transgenic rice line (164-12) expressing high levels of recombinant human LF was selected. This line. now named as LF164, was planted two generations per year, alternating field planting in summer and greenhouse planting in winter. For protein purification, paddy rice expressing rhLF was de-hulled (Rice Mill, PS-160, Rimac, FL), and then ground to flour (average particle size of 100 mesh) using a hammer mill (8WA. Schutte-Buffalo, NY).

Protein extraction from transgenic flour was performed by mixing two kg of rice flour and 20 L of extraction buffer (0.02 M sodium phosphate pH 6.5 and 0.3 M sodium chloride) in a 50 L tank for 1 h. At the end of the mixing period, the suspension was allowed to settle overnight or centrifuged at 3750 rpm. In both cases, the supernatant was filtered through a plate and frame filter press (Ertel Alsop, 8S. NY) using M-05 and M-70 cellulose/perlite-based filters (Ertel Alsop. NY), respectively.

The filtrate containing rhLF and other rice flour soluble proteins was loaded onto an ion exchange column for further purification. An INDEX 200/500 process column (Amersham Pharmacia Biotech, NJ) packed with SP-Sepharose fast flow (Amersham Pharmacia Biotech, NJ) was used. The column was run at a linear flow rate of 150-200 cm/h. Packing, cleaning and testing of the resin bed was executed per manufacturer's instruction (HETP Test). The filtrate was loaded on to the resin at a linear velocity of 175 cm/h and washed with 0.02 M sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl until the A_{280} returned to baseline. Recombinant hLF was eluted using 20 mM sodium phosphate buffer (pH 6.5) containing 0.8 M NaCl. The washing and elution were performed at 200 cm/h and 150 cm/h, respectively.

A Centramate module (Pall Biopharmaceutical, MA) with 1 ft^2 50 kDa polyethersulfone (Pall Biopharmaceutical, MA) membrane was used for concentration and desalting (ultrafiltration) of eluted hLF. The filtration was performed at a cross flow rate of approximately 1.5 L/min and an average transmembrane pressure of 10 psig. The eluted rhLF was concentrated and desalted to a final volume of 0.25 L and then lyophilized dry. Usually, about 3 grams of purified recombinant human lactoferrin was recovered from one kilogram of transgenic rice flour.

The recombinant human lactoferrin purified from rice flour is approximately 50% saturated with iron (partial-lactoferrin). The 50% saturated recombinant human lactoferrin was then made >90% iron saturated by iron up taking treatment, resulting in holo-lactoferrin and was made <10% iron saturated by acid treatment to remove bound iron resulting in apo-lactoferrin. The purified lactoferrins (holo-, partial- and apo-) were used as cell culture media components.

Example 5: Purification and characterization of recombinant serum human albumin (rHSA).

Overview of B000 production process.

To purify rHSA from rice grain, rice grain expressing recombinant human serum, as described above, was dehusked, ground to flour and mixed for 30 min. with rHSA extraction buffer (Sodium Acetate, pH 4.9). After clarification by filtration, the filtrate was loaded onto an anion-exchange column (Q-Sepharose; GE Healthcare) at a rate of approximately 150 cm/hr. Once loaded, the resin was then washed with equilibration buffer to remove any unbound protein. The eluted rHSA is approximately 90% pure as analyzed by SDS-PAGE. Blue-Sepharose (GE Healthcare) can then be used to increase the purity from 90% to approximately 98% based on scanning analysis of SDS-PAGE gels. The rHSA is then concentrated and desalted via ultrafiltration prior to freeze drying.

Purified recombinant HSA was subjected to a range of biochemical and biophysical characterizations for comparison to pHSA (Plasma-derived HSA) (**Table E2**). These tests indicate that rHSA expressed in rice is equivalent to pHSA

Table E2

Properties of recombinant albumin (rHSA) as compared to plasma derived albumin (pHSA)

Property	pHSA	rHSA
N-terminal sequence	DAHKSE	DAHKSE
Glycosylation	None	None
SDS-PAGE/Western	~66 kDa	~66 kDa
Molecular mass (MALDI)	66.9-68.1 kDa	66.8-67.9 kDa
Isoelectric focusing point	pI 5.3	pI 5.3
Ligand binding	Yes	Yes
Thermal stability	Midpoint 65°C	Midpoint 65°C
Esterase activity	Yes	Yes
Protease sensitivity	Same as each other	Same as each other

B0000C Production Process: For Ventria grown rice, the rice was harvested by combine or by hand. During this process the mature seeds were separated from the vegetative plant matter by the combine separator or by manual labor. The harvested rice was dried to approximately 12% moisture at which point it is suitable for storage in a clean grain bin, storage tote, supersack, or other container that will protect the grain from birds, rodents, lizards, insects and other pests. When the rice grain is needed for flour, it is first dehusked or dehulled. This process is done under vacuum such that debris and the outer part of the seed are swept away from the endosperm and germ or bran layer. The dehusked grain is then either washed and dried, or washed and processed directly as in wet homogenization, or processed further in the dry, dehusked state. The dry, dehusked material may be debranned by a rice polishing or debranning machine which are common to white rice producers.

Debranned, dehusked rice may be washed at this point and wet-milled or dried for dry milling or processed directly by grinding into flour. Milling with the least amount of shear and heat is preferred as such with a roller mill or pin mill. A hammermill is also suitable. The flour should be ground such that the protein can be extracted to 90% in less than 5 minutes in water with hard agitation. Normally that requires a size of particle that is smaller than 400 micrometers or 4 mm. However, larger particles can be extracted if given longer time. Alternatively, the grain can be washed and wet milled with a liquid homogenizer set up such that 90% of the extractable protein is solubilized.

The flour slurry is typically mixed at a ratio of at least 3 parts water to 1 part flour and up to 20 parts water to 1 part flour. The water typically contains suitable buffers such as Tris/HCl. Citrate, Phosphate, HEPES, or the like, such that the pH is maintained around pH 7 and a small amount of salt such as 100 mM NaCl. After the slurry is homogenized in the case of wet milling, or mixed thoroughly for dry flour, the bulk solids are removed from the slurry by way of solid liquid separation. This is carried out by decanting, centrifugation, or filtration; for example using plate and frame with pads, pressure filter, belt filter, vacuum flask, hydroclone, or vacuum belt filter. After filtration, the compressed cake should be washed with extraction buffer to recover protein from the cake. The addition of diatomateous earth or other filter media is useful in promoting the clarity of the filtrate but is not necessary given the right equipment. Alternatively, a flocculating agent may be used to aid in clarification. The clarified filtrate should be checked for its albumin content and verified that the recovery is consistent with the determined expression level in the rice seed.

In order to remove starches, precipitable proteins, viruses, and other contaminants, 5 M acetic acid is added to the clarified filtrate until the pH reaches 5.0 and the solution turns white. The white solution is agitated for at least 20 minutes to encourage precipitation of insoluble materials. The precipitated solution is then filtered through a depth filter, such as a canister filter, cartridge filter or other filtration device to reach an optical clarity that is suitable for ultrafiltration, or less that 10 NTU (nephelometry turbidity units). It can also be clarified with a filter press, pressure filter, or alternatively by using a ceramic filter or other material that utilizes cross-flow. In addition, this material is suitable for direct application to an expanded bed chromatography column.

In a preferred method, the clarified filtrate is clarified via filtration through a 0.2 micron filter, and neutralized to pH 7.0 with 1M NaOH. This material is then suitable for ultrafiltration by hollow fiber, flat sheet, or spiral wound cross flow filtration. The material can be passed through a membrane of 100 kilodalton (kDa) size or larger to remove viruses, unwanted larger contaminants, and aggregates. The material that passes through the membrane can be concentrated by a 10 or 30 kDa crossflow membrane and then the same membrane can be used to prepare the solution for chromatography. The concentrated material can then diafiltered with column equilibration buffer until the conductivity and the pH are equalized.

The preferred buffer for anion exchange chromatography on GE DEAE sepharose or GE Q sepharose is 10 or 20 mM Tris/HCl buffer pH balanced to pH 8.0. In contrast, the preferred buffer for cation exchange, for example via the use of for negatively charged resins or

negatively charged resins mixed with a hydrophobic linker (mixed mode absorbents), or alternatively blue Cibicron such Blue Sepharose (GE) is acetate or citrate buffer pH balanced to 4.8 to 5.0

For either system the albumin and other similarly charged proteins will be retained by the matrix and washing is conducted to remove loosely bound material by washing with at least 5 column volumes of loading buffer, which may also include detergents as deemed necessary to help remove hydrophobic impurities. The material can be eluted by charging the column with the same or modified buffers with the pH increased 2-4 units for cation exchange or decreased 2-4 units for anion exchange. The resulting change in pH will allow for the exchange of ions and the protein will be eluted in a sharp band. To increase the purity of the elution fraction, the elution peak can be scrutinized such that the first portion (10%) or last (10%) or both portions can be excluded from the main elution peak. In the preferred method, a solution containing phosphate at 100 mM and pH adjusted to pH 4.0 including 10 mM NaCl is used to elute the protein from GE Q sepharose (Fast Flow). In this instance, pH and conductivity are used to elute the material allowing the discrimination between non-binding contaminants (flow through and wash) and tighter binding contaminants (those that are retained on the column in 100 mM Phosphate, 10 mM NaCl, and pH adjusted to 4.0).

After elution, if the pH of the eluted material has a pH of less than 6.0, then it is neutralized with 1M NaOH. The resulting solution is then diafiltered against the same buffer for the next chromatography step, which in a preferred method involves flowing the elutent through a column of the same matrix (i.e. Q sepharose) except in the non-binding mode with 100 mM Phosphate, 10 mM NaCl, and pH 7.0.

The second column step uses the same principles as the first but in reverse mode such that the contaminants that were co-eluted on the binding column have an opportunity to be retained on the matrix at a neutral pH. The flow through material from the first capture column can also be treated with a variety of alternative types of chromatography approaches, for example, cation exchange, hydrophobic, mixed mode, or gel filtration chromatography.

In a preferred method, the flow through material from the Q sepharose non-binding column is concentrated on a 10 kDa or 30 kDa crossflow membrane until the concentration is between 15 and 25% albumin. The buffer is then changed by diafiltration into a suitable buffer for cell culture such as Dulbeccos PBS or alternatively 20 mM Phosphate, 50 mM NaCl, and pH 7.0. The material is then sterile grade filtered into a sterile container. The sterile filtered material

may be treated with detergent to destroy enveloped viruses and to aid in the removal of hydrophobic toxins and contaminants. In a preferred method, 0.5% v/v Triton X-114 or X-100 is added to the 15 to 25% albumin solution at room temperature (less than 23 C and greater than 18 C) and the solution is agitated or stirred for at least hour. The material is then passed over a hydrophobic resin with a molecular weight exclusion limit that is much less than the molecular weight of albumin. Many commercially available resins are available including those from Biorad and Pall Corporation

The material that is passed over the column may then be tested in cells that are sensitive to detergent to confirm biological activity. The residual detergent that remains should typically be less than 0.005% with respect to the albumin solution. The detergent free flow through can then be sterile filtered into containers for direct shipment, or can have stabilizers added, or can be subjected to pasteurization with stabilizers, or can have stabilizers added before drying or dried directly. The material may be dried by lyophilization or spray drying. Prior to drying, in some instances, it may be useful to subject the material to a virus filtration step using a disposable, validated, virus removing capsule such as is available from GE, Pall, and Millipore. It is common in the art to understand that a prefiltration step may be necessary in order to effectively and economically pass the concentrated material through a 20 nm filter.

Results: Rice flour was extracted at 1:5 ratio in phosphate buffered saline and mixed for 20 minutes. The liquid was clarified using a Nalgene filter flask The subsequent clarified extract was subjected to acid precipitation as is described in the methods. The solution was then filtered and neutralized to give a clarified filtrate. This material was diafiltered against 50 mM Tris/Cl pH 8.0 until the material and buffer were equilibrated. The material was then loaded (300-600 cmh) on a pre-equilibrated GE Q-sepharose column to allow for 50 g/L binding capacity The loaded material was washed with the same buffer and the material was then eluted with 100 mM Phosphate, 10 mM NaCl, and pH 4.0 as described above. The material eluted in a sharp peak and the collected eluate had a stable pH of about 5.8. Albumin produced using this method was compared to other sources of Albumin as more fully disclosed below. The eluate was collected in a pool and 1M NaOH was added until the pH was greater than 6.0. The material was then concentrated on a 10 kDa regenerated cellulose membrane approximately 5 fold and approximately five equal volume diafiltrations were carried out with 100 mM phosphate, 10 mM NaCl, pH 7.0. The final diafiltered material was checked for albumin protein content (in relation to the expression level in the starting material should be greater than 80%) and endotoxin level (should be less than 100 EU/mg depending on the feed material). This material was passed (60-160 cmh) over a Q-sepharose column, equilibrated with 100 mM

phosphate, 10 mM NaCl, pH 7.0, of sufficient size to allow for approximately 2-3 times loading volume. The material was washed through the resin with the same buffer and collected. The collected material was diafiltered on a 10 kDa regenerated cellulose membrane and concentrated approximately 10 fold or until the albumin concentration reaches at least 10% or not more than 20% and five equal volume diafiltrations were performed with 20 mM phosphate, 50 mM NaCl, pH 7.0. After sterile grade filtration (0.2 µm), the solution was agitated for 1 hour with 0.5% (v/v) Triton X -100 at 20 +/- 2°C. After the incubation, the material was passed through Pall SDR resin according to the manufacturer's directions The flow through material was sterile grade filtered into sterile containers and refrigerated or freeze dried as is common for protein and salt solutions.

Example 6: Comparison of recombinant albumin produced from rice using B0000C process compared to other sources of albumin and previous methods for the production of Albumin.

Methods: Albumin prepared using process B000C described in Example 5, was compared to albumin prepared using an alternative process (B000) which was previously used to prepare recombinant albumin (Cellastim (Batches B202 to B217)).

Albumin production (old process, B000):

Rice flour and 25 mM Sodium phosphate, 50 mM Sodium Chloride, was pH balanced to 6.5 with NaOH and mixed for 20 minutes at room temperature with a S/L ratio of approximately 1.10. Filter aid (Cellpure 300) was added at 10 g/L and the slurry was filtered by filter press, vacuum filtration, or centrifugation. The clarified filtrate was acid precipitated to about pH 5.0 with 1 M acetic acid. The resulting solution was filtered as described above with the addition of 5 g/L filter aid (Cellpure 300). The material was neutralized immediately to pH 6.5 to 7.0 with 1M NaOH. The material was diafiltered (10 kDa regenerated cellulose for all UFDF steps) with 5 equal diavolumes of the same buffer used for extraction. The material was loaded on a pre equilibrated Q-sepharose column (GE Healthcare) to allow for 8 g albumin binding per liter of resin at 60 cmh.

After washing the column with 5 column volumes of the same buffer, the albumin was eluted by increasing the salt concentration to 250 mM NaCl in one step. The resulting material was diafiltered against 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0 with 5-7 equal diavolumes. The resulting material was passed over a Q-sepharose column equilibrated with the 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0, and collected as flow-through. The flow-through

material was then concentrated and diafiltered against 20 mM sodium phosphate, 10 mM NaCl, pH 7.0 with 5 diavolumes. The final concentrated material was sterile filtered and incubated with 10 g/L of the detergent CHAPS ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid) and mixed at room temperature for 1 hour. After the one hour incubation, the material was passed over a Biorad SM-2 column. The material was sterile filtered and freeze dried.

Size Exclusion Chromatography analysis.

Purity analysis by HPLC was carried out in 100 mM phosphate, pH 7.0 on a GF-250 column (Agilent Technologies) at a flow rate of 1 ml/min with the detector set at 214 and 280 nm. A standard curve was developed by injecting 5 different dilutions made by dry powder with a correction factor of 0.92 for salt and moisture. The main peak from 214 nm was integrated either by retention time or alternatively baseline The unknown sample was injected at a concentration that is within the range of the standard injections. The unknown concentration of albumin per dry powder weight (purity) was calculated from the standard curve. In a typical experiment, the 0, 5, 8, 10, 15, and 20 µg of the standard was injected followed by approximately 10 µg of unknown sample in approximately 50 µL injection volume. The correlation coefficient for the standard curve after integrating the peaks was typically above 0.98.

SDS PAGE and Densitometry: (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Samples were prepared by diluting the protein solutions to 1-2 mg/ml to enable a defined amount of each protein to be loaded on to each well. The sample was mixed 1:1 with Tris-Glycine SDS sample buffer (LC2673 Novex) containing reducing agent (Invitrogen NP0004) and heated to 70 °C for 5 minutes. The sample was loaded (10, 20, or 30 µg) onto a Novex 4-20% precast gel and separated at constant voltage (130V) in standard Tris-Glycine-SDS running buffer. The electrophoresis was ended when the tracking dye reached the end of the gel. A molecular weight marker was included in the first lane as a reference.

The gel was stained with G Bioscience (786-35G) and destained with water A digital image was obtained with a Hewlett Packard Scanner (G4010). The image file was then opened with UN-SCAN-IT (Silk Scientific Corp.). The densitometry was carried out with positive image analysis in 256 grayscale in which all visible bands were included as individual segments. The background noise was corrected by four corner interpolation as specified in the software for each segment. The signal for each segment or band was then calculated from the product of the # of pixels and the average pixel intensity (0-255). The sum of the signals for an entire lane (all visible segments or bands) was taken as 100% and the impurity bands were subtracted to

calculate the albumin purity. The percent of each contaminating protein in each band was calculated as the number of peptides identified for that contaminant protein as determined by peptide mapping divided by the total number of all peptides identified in a particular band. The image analysis was repeated 3 times such that the standard deviation is less than 0.5% out of 100%

Determination of endotoxin by the Pyrogene rFC method.

Endotoxin content was determined by the Pyrogene rFC method Lyophilized endotoxin standard was mixed with endotoxin free water as specified by the manufacturer (Lonza) to develop a standard curve. The protein samples were either diluted as is for liquid or alternatively, reconstituted with endotoxin free water for powder. Different dilutions were prepared such that the readings should appear within the range of the standard curve. The samples were heated to 100 °C for 10 minutes to dissociate unwanted molecular interactions. In a typical experiment, the sample and standard were added at 100 µl per well, with 0, 0.001, 0.005, 0.01, 0.05, and 0.1 endotoxin units per well. The samples were also added at 100 µl and extra samples were included such that spiking with 0.001 -0.01 endotoxin units per well were added to test for assay inhibition or interference. The working reagent was prepared according to the manufacturer (Lonza) by mixing the rFC enzyme, assay buffer, and substrate in a 1:4:5 ratio, respectively. The working reagent was added to the wells at equal volume to the sample or standards. The fluorescence plate reader (Biotek FLX 800T) was set for excitation at 380 nm (bandwidth =20 nm) and the emission wavelength was set at 440 nm (bandwidth =30 nm) The reading taken at time zero is subtracted from the reading taken after 1 hour at 37 °C. The readings were considered valid if the correlation coefficient, slope, and Y-intercept for the standards was within the set limits, and the spiking experiments show that the spiked endotoxin was measureable and recoverable within the set limits In addition, the standard deviation for duplicate samples should be in reasonable agreement such that the standard deviation was within a specified arbitrarily chosen limit. All samples were collected aseptically and the tubes/vials/containers used for testing were verified to be extremely low endotoxin following good laboratory practices as they relate to accurate and precise endotoxin testing.

Determination of Cell Viability

The hybridoma cell line AE1 (ATCC) was maintained in DMEM basic media containing 5% fetal bovine serum (FBS). Albumin was tested under serum-free conditions (AFM6, KC Bio, Kansas) without supplementation of fetal bovine serum. The cells were subculture from 5% FBS to serum free media over multiple passages. At each subculture, the cells were analyzed for total cell count and viability in the presence of the indicated concentrations of albumin (As assessed

by trypsinization and direct counting using a Neubauer haemocytometer). The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Date show the number of viable cells / ml divided by 10^5.

Determination of Detergent

The detergent concentration for the albumin was determined by a detergent (cell based) assay. Briefly, detergent sensitive cells were spiked with different amounts of detergent and the resulting cell viability cell determination used to generate a standard curve consisting of 16 independent data points. The change in viability with respect to the change in detergent concentration was plotted and fitted with a logarithmic function. This equation was then used to calculate the unknown detergent concentrations in samples tested in the same cell based assay. The correlation coefficient for the standard curve for the data given was 0.9816. Typically detergent concentrations of greater than about 10 ppm per Cellastim dry weight, result in noticeable toxic activity. By comparison in a 10% albumin solution, toxic effects of detergent become apparent when the detergent concentration is above about 100 ppm to 200 ppm or 0.01% to 0.02 % (v/v).

Results & Discussion:

I. Analysis by Size Exclusion chromatography of plasma derived serum (Sigma Albumin), and recombinant HSA (Cellastim) produced using the new process (B000C) (Cellastim P0171) and the old process (Cellastim P0107).

The HPLC size exclusion profiles (**FIG. 8A, C & D**) for the three types of albumin show that in terms of overall purity the different albumin preparations are generally similar. Specifically, the peaks at around 4.5 kDa and 240 kDa are the internal controls, while all three products contain a very small amount of an off main peak signal at about 10-12 kDa.

Human plasma derived albumin (Sigma Albumin) (**FIG. 8A**), contains a contaminant at around 17 kDa, by comparison the recombinant rice derived albumin using the new process (Cellastim P0171) (**FIG. 8C**) contains two protein contaminants of around 44 KDa and 55 kDa that occur in Cellastim made with the new **FIG** at significantly higher levels than when using the old process (Cellastim P0107) (**FIG. 8B**). These peaks are not completely resolved in the HPLC separations, but can be seen as more clearly in the overlaid profiles of Cellastim P0171 and Sigma albumin (**FIG. 8D**) and Cellastim made using the old and new processes shown in **FIG. 8E.**

The proteins corresponding to these peaks represent about 5% of all of the contaminant proteins identified by Peptide Mass Fingerprinting analysis of the main albumin peak in Cellastim produced using the process described in Example 5, as discussed further below

All albumin products tested also contained a peak at around 130 kDa that most likely represents albumin dimers, it is noticeable that the Cellastim dimer peak is significantly smaller than the plasma derived albumin The creation of aggregated albumin is an indicator of protein degradation which is used as one marker for degradation or loss of stability industry wide. It is likely that the Hsps present in Cellastim promote the disaggregation of the albumin, therefore reducing the number of dimers, since it is a commonly known function of Hsp 70 and other Hsp proteins

II Analysis by SDS PAGE of Cellastim batch **P0171** (New process) compared to albumin produced by Millipore/Novozymes (Cat No. 9301-01)

Results: SDS-PAGE analysis (**FIG. 9A & FIG. 9B**) shows that in terms of overall purity the products are generally similar. **FIG. 9A** shows a comparison of Cellastim P0171 and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **FIG. 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

Visual inspection of the gel shows that the new process which meets more rigorous specifications is more consistent among the 3 lots tested. (**FIG. 9B**, lane 2, 3, 4 vs. lane 6, 7, 8). The banding pattern is significantly different among the three samples from the previous process as compared to the new process. Importantly, the new process samples have significantly less aggregates at around 250 KDa than the old process samples have. (Average greater than 2 % for the old process, and average less than 1 % for the new process). The identity of the protein contaminates was that are enriched in Cellastim produced using the new process is discussed further below.

III. Analysis of endotoxin, detergent and growth promoting abilities of old and new batches of Cellastim. A comparison of the performance of the two different processes for preparing several different lots of albumin (**Tables E3 and E4**) demonstrates that the old

process produced recombinant albumin that contained significantly more endotoxin, and detergent compared to the new process described in Example 5, and resulted in a product that significantly enhanced cell viability.

Table E3

Cellastim - Old Process B000

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					1mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/27/08	B202	70.6	25.3	815 3	15	7.9	4.1	0.9
2/27/08	B203	58.8	23.1	267.7	15.8	12.4	6.5	4.1
2/28/08	B204	59.9	29.2	589.2	16.3	11 1	5.2	2.9
2/28/08	B205	63	64.3	133.4	14	11.6	6.9	3.6
3/3/08	B206	41.1	35.1	1182.2	14.9	2 3	0.0	0.0
3/3/08	B207	94.2	28.7	388	16.5	12.7	7.6	4
3/4/08	B208	24.5	>80	467.2	15 3	11.4	7.1	2.7
3/4/08	B209	66.1	91.8	406.4	16.7	15.2	6.9	4.1
3/4/08	B210	87.3	67 8	76.4	17.5	19.1	15.4	8.5
3/11/08	B217	62.09	4.4	127.3	16.7	18.7	13.2	6.6
Averages			**44.97**	**445.31**	**15.87**	**12.24**	**7.29**	**3.76**

Table E4

Cellastim - New Process B0000C

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					0.5mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/2/2009	B0032C	209 4	0 35	3 1	13.1	15.7	13.4	11 2
2/2/2009	B0033C	271.4	0.26	7.9	18.4	17.4	15.1	14.3
2/10/2009	B0041C	247.1	0.18	5.4	15.4	17 2	14.1	12.1
4/27/2009	B0118C	617.4	0.11	Less than 1	15.7	15.4	19.2	16.8
5/14/2009	B0138C	610 6	0 48	Less than 1	12.5	15.8	15.1	14.6
6/9/2009	B0158C	598.0	0.13	3.9	13.7	17.1	13.2	11
6/15/2009	B0162C	618.6	0.20	2.0	15.5	17 5	17.2	14.7

7/28/2009	B0196C	507.0	0.36	3.9	17.4	17.3	18.8	14.8
8/26/2009	B0219C	851.4	0.86	8.2	17.9	16.3	16.6	13.7
8/31/2009	B0220C	897.8	0.93	Less than 1	13.5	16	15.5	15.1
9/9/2009	B0227C	929.9	0.13	Less than 1	11.1	14.4	12.5	11.4
Averages			**0.36**	**3.45**	**14.92**	**16.37**	**15.52**	**13.6**

Discussion: Re-engineering the old process to create the new process described in Example 5 resulted in significant changes in both overall product purity, and performance, as described more fully below.

The changes made it possible to make products that were lower in detergent, lower in endotoxin, and increased purity. Specifically, the new process routinely produced recombinant albumin with an overall purity of greater than about 95%. By comparison the old method routinely produced albumin with a maximum purity of about 90 %. Surprisingly, despite the increased product purity, these changes in processing also resulted in enhanced co-purification of heat shock proteins, (see below) with the recombinant albumin. Without being bound by any particular theory of operation, it is believed that the combination of high albumin purity, relative lack of endotoxin and / or detergent, and co-purification of heat shock proteins results in a product that significantly out performs previous methods for preparing albumin.

Specifically **Tables E3** and **E4** demonstrate that the new process for producing recombinant albumin results in a product that, for example at 5 mg /ml, results in an average batch to batch 100 percent improvement in cell viability (at 5 mg /ml), and also results in a product with an average 100-fold less endotoxin, and 100 fold less detergent than the old process.

Example 7: Analysis of the effects on cell growth and viability.

To compare the cell growth promoting abilities of the recombinant albumin prepared above, to other commercially available albumin products, the different sources of albumin they were compared side by side in a cell growth and viability assay. The three products tested were (Cellastim, Lot # P0153) Celiprime albumin (Millipore/Novozymes Cat No. #9301-01), and plasma derived albumin (Seracare Cat No. #HS-400-60).

Methods: Specially conditioned Hybridoma cells AE1 were seeded in DF12/ITSE at a density of 0.5×10^5 cells per ml of media after washing twice with same media to remove residual media.

The media and cells were then left untreated (negative control), treated with Seracare albumin, treated with Cellprime albumin, and treated with rice derived recombinant albumin at the concentrations shown in the figure legend. The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Results are shown in FIG. 10A-FIG. 10C.

Results: Novazyme's Cellprime albumin caused a loss in viability (cross-hatch bars). Seracare albumin (white bars) caused a measureable increase in viability but not as large an increase as is seen with recombinant albumin made in rice with the new process (Cellastim) (black bars). Under these conditions the rice derived recombinant albumin was approximately 5 times as active in promoting cell viability compared to any of the other albumin products, at any concentration tested. The negative control is represented by the striped bars.

Discussion: Given the possibility that the other commercially available albumin products may have similar overall purity, endotoxin and detergent levels to rice derived recombinant albumin, the dramatically superior performance of the recombinant rice derived albumin compared to other commercially available albumins suggests that the previously un-identified protein contaminants identified in Cellastim compared to the plasma derived albumin (Example 5) could be having a positive impact on cell viability. To identify and then characterize the impact of these proteins on the properties of Cellastim, a sample of the recombinant albumin was subjected to peptide mass finger printing, as described below.

Example 8: Peptide Mass Finger printing of recombinant human serum albumin.

Methods: Samples of albumin were analyzed to determine significant protein contaminants using a NanoLCMS/MS peptide sequencing system (ProtTech, Inc.), and proprietary software to identify the proteins based on the molecular weight of the peptide fragments. In brief, samples of albumin were analyzed by SDS-PAGE, and each major band gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin. The resulting peptide mixture was analyzed by a LC-MS/MS system, in which a high pressure liquid chromatography (HPLC) with a 75 micrometer inner diameter reverse phase C18 column was used in-line coupled with an ion trap mass spectrometer. The mass spectrometric data acquired was used to search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting

Results: Upon testing of three representative lots of recombinant albumin, three Hsp70 proteins were identified by Peptide Mass Fingerprinting (**Table E5**) The three specific sequences identified: ABF95267, ABA97211, and BAD 07938 were compared to the non redundant database to identify highly related and homologous proteins. The results of the top hits from each of these comparisons is shown in **Tables E6, E7 and E8** below.

Table E5

Peptides identified from Cellastim by mass finger printing

Sequence	Peptide
ABF95267	ATAGDTHLGGEDFDNRVVPGPADKSPMIVVTYKGEEK NAVITVPAYFN DSQRIINEPTAAAIAYGLDKK (**SEQ. ID. No. 2**)
AAB63469 BAD07938 BAD07713	NQAAVNPER NGHVFIIANDQGNRIVNKDGKPYIQVK IINEPTAAAIAYGLDKK KLGTVIGIDLGTTYSCVGVYK VEIESLFDGTDSFSEPLTR (**SEQ. ID. No. 3**)
ABA97211	NQADSVVYQTEK KQDITITGASTLPKDEVERDVVLLDVTPLSLSLGLET LGGVMTK (**SEQ. ID. No. 4**)

Results of sequence comparisons to ABF95267 sequences in the non redundant database of protein sequences in Genbank® (Nucleic Acids Research, 2008 Jan;36(Database issue):D25-30) are shown in **Table E6** below.

Table E6

Sequences producing significant alignments with ABF95267:

Gene Refs	Gene description	(Bits)	Value
ref|NP_001140835.1|	hypothetical protein LOC100272911 [Zea ma...	79.7	8e-14
ref|XP_002465468.1|	hypothetical protein SORBIDRAFT_01g039390...	79.7	9e-14
ref|NP_001049719.1|	**Os03g0277300 [Oryza sativa (japonica cult...**	**79.7**	**9e-14**
gb|ACJ54890.1|	heat shock protein 70 [Oryza sativa Japonica G...	79.7	9e-14
Sp|P09189.1|	HSP7C_PETHY RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
emb|CAA31663.1|	hsp70 (AA 6 -- 651) [Petunia x hybrida]	77.0	5e-13
ref|XP_002312089.1|	predicted protein [Populus trichocarpa] >...	77.0	5e-13
sp|P24629.1|	HSP71_SOLLC RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
gb|AAB99745.1|	HSP70 [Triticum aestivum]	76.6	6e-13
gb|AAB42159.1|	Hsc70 [Lycopersicon esculentum]	76.6	7e-13
gb|ACD45076.1|	heat-shock protein 70 [Dactylis glomerata]	76.3	8e-13
ref|XP_002512741.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
ref|XP_002512742.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
gb|AAA82975.1|	PsHSP71.2 >emb|CAA67867.1| heat shock protein ...	75.9	1e-12
gb|AAS09825.1|	heat shock cognate protein 70 [Thellungiella h...	75.9	1e-12
emb|CAA44820.1|	heat shock protein 70 [Nicotiana tabacum]	75.5	2e-12
ref|NP_001055754.1|	Os05g0460000 [Oryza sativa (japonica cult...	75.5	2e-12
ref|NP_001051724.1|	Os03g0821100 [Oryza sativa (japonica cult...	75.1	2e-12
ref|XP_002456611.1|	hypothetical protein SORBIDRAFT_03g039360...	75.1	2e-12
ref|NP_001044757.1|	Os01g0840100 [Oryza sativa (japonica cult...	75.1	2e-12
gb|ACR35910.1|	unknown [Zea mays]	75.1	2e-12
ref|XP_002284017.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002532297.1|	heat shock protein, putative [Ricinus com...	75.1	2e-12
ref|XP_002284008.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002283532.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.7	3e-12
ref|XP_002332067.1|	predicted protein [Populus trichocarpa	74.7	3e-12
gb|AAF34134.1|	high molecular weight heat shock protein [Malu...	74.7	3e-12
gb|EEC76425.1|	hypothetical protein Osl_14101 [Oryza sativa I...	74.7	3e-12
ref|XP_002316294.1|	predicted protein [Populus trichocarpa] >...	74.7	3e-12
ref|XP_002283516.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.3	3e-12
ref|XP_002441219.1|	hypothetical protein SORBIDRAFT_09g022580...	74.3	4e-12

Results of sequence comparisons of ABB63469 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E7** below:

Table E7

Sequences producing significant alignments with AAB63469:

Gene Refs	Gene description	(Bits)	Value
emb|CAP31983.1|	C. briggsae CBR-HSP-4 protein [Caenorhabditis...	50.1	7e-05
dbj|BAG60366.1|	unnamed protein product [Homo sapiens]	49.7	8e-05
ref|YP_002421952.1|	chaperone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|YP_001640420.1|	chaperone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|NP_001105893.1|	Binding protein homolog1 precursor [Zea m...	49.3	1e-04
gb|AAA62325.1|	HSP70	49.3	1e-04
ref|YP_001756576.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
gb|AAB63469.1|	endosperm luminal binding protein [Oryza sativa]	49.3	1e-04
ref|YP_001925829.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
ref|NP_001105894.1|	Binding protein homolog2 precursor [Zea m...	49.3	1e-04
gb|ACF86491.1|	unknown [Zea mays]	49.3	1e-04
ref|NP_001045675.1|	**Os02g0115900 [Oryza sativa (japonica cult...**	49.3	1e-04
ref|ZP_02191025.1|	Molecular chaperone (alpha proteobacterium...	49.3	1e-04
ref|XP_001701685.1|	binding protein 1 [Chlamydomonas reinhard...	48.5	2e-04
ref|XP_001701884.1|	binding protein 2 [Chlamydomonas reinhard...	48.5	2e-04
emb|CAC37635.1|	luminal binding protein, BiP [Scherffelia dubia]	48.1	3e-04
gb|AAM93256.1|	heat shock protein 70-C [Heterodera glycines] ...	48.1	3e-04

Results of sequence comparisons of sequence ABA97211 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E8** below:

Table E8

Sequences producing significant alignments with ABA97211

Gene Refs	Gene description	(Bits)	Value
gb|AAK13022.1|	heat shock protein 70 [Fibrobacter succinogene...	44.3	0.004
ref|XP_002442079.1|	hypothetical protein SORBIDRAFT_08g009580...	44.3	0.004
gb|EEC69073.1|	**hypothetical protein OsI_37938 [Oryza sativa I...**	**44.3**	**0.004**
ref|XP_001752769.1|	predicted protein [Physcomitrella patens ...	44.3	0.004
ref|NP_001066486.1|	Os12g0244100 [Oryza sativa (japonica cult...	44.3	0.004
gb|ACT65562.1|	70 kDa heat shock protein [Triticum aestivum]	43.9	0.005
ref|NP_001152528.1|	stromal 70 kDa heat shock-related protein...	43.9	0.005
gb|ACN31310.1|	unknown [Zea mays]	43.9	0.005
ref|XP_001772650.1|	predicted protein [Physcomitrella patens ...	43.9	0.005
ref|NP_001146752.1|	hypothetical protein LOC100280354 [Zea ma...	43.9	0.005

gb|ABP65327.1|	chloroplast heat shock protein 70 [Pennisetum ...	43.9	0.005
gb|AAO72585.1|	heat shock-related protein [Oryza sativa (japo...	43.5	0.007
ref|YP_001740846.1|	Chaperone protein dnaK (Heat shock protei...	43.5	0.008
ref|ZP_03728467.1|	chaperone protein DnaK [Dethiobacter alkal...	43.1	0.009

Discussion: Peptide Mass Fingerprinting identified 3 rice heat shock protein super family members that co-purify with albumin, 2 Rice HSP70 genes, (gb|ACJ54890.1|), EEC69073, and AAB63469 - a BiP homolog from rice endosperm tissue (endosperm lumenal binding protein). The complete amino acid sequences coded by these genes are listed below:

Gene gb|ACJ54890.1| heat shock protein 70 [Oryza sativa Japonica Group] HSP70 was found to occur in recombinant albumin in Cellastim at approximately 0.07% wt / wt. Its complete amino acid coding sequence (**SEQ. ID. No. 5**) is provided below:

MAGNKGEGPA IGIDLGTTYS CVGVWQHDRV EIIANDQGNR TTPSYVAFTD TERLIGDAAK
NQVAMNPTNT VFDAKRLIGR RFSDPSVQAD MKMWPFKVVP GPADKPMIVV TYKGEEKKFS
121 AEEISSMVLT KMKEIAEAFL STTIKNAVIT VPAYFNDSQR QATKDAGVIS GLNVMRIINE
181 PTAAAIAYGL DKKAASTGEK NVLIFDLGGG TFDVSILTIE EGIFEVKATA GDTHLGGEDF
241 DNRMVNHFVQ EFKRKHKKDI TGNPRALRRL RTACERAKRT LSSTAQTTIE IESLYEGIDF
301 YATITRARFE ELNMDLFRRC MEPVEKCLRD AKMDKAQIHD VVLVGGSTRI PKVQQLLQDF
361 FNGKELCKSI NPDEAVAYGA AVQAAILSGE GNQRVQDLLL LDVTPLSLGL ETAGGVMTVL
421 IPRNTTIPTK KEQVFSTYSD NQPGVLIQVY EGERTRTKDN NLLGKFELTG IPPAPRGVPQ
481 INVTFDIDAN GILNVSAEDK TTGKKNKITI TNDKGRLSKE EIERMVQEAE KYKAEDEQVR
541 HKVEARNALE NYAYNMRNTV RDEKIASKLP ADDKKKIEDA IEDAIKWLDG NQLAEADEFE
601 DKMKELESLC NPIISKMYQG GAGGPAGMDE DAPNGSAGTG GGSGAGPKIE EVD

AAB63469 BiP homolog from rice endosperm tissue (endosperm lumenal binding protein [Oryza sativa]) BiP was found to occur in recombinant albumin in Cellastim at about 0.09% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 6**) is provided below:

mdrvrgsafl lgvllagslf afsvakeetk klgtvigidl gttyscvgvy knghveiian
dqgnritpsw vaftdserli geaaknqaav npertifdvk rdigrkfeek evqrdmklvp
121 ykivnkigkp yiqvkikdge nkvfspeevs amilgkmket aeaylgkkin davvtvpayf
181 ndaqrqatkd agviaglnva riineptaaa iaygldkkgg eknilvfdlg ggtfdvsilt
241 idngvfevla tngdthlgge dfdqrimeyf iklikkkysk diskdnralg klrreaerak
301 ralsnqhqvr veieslfdgt dfsepltrar feelnndlfr ktmgpvkkam ddagleksqi

```
361 heivlvggst ripkvqqilr dyfegkepnk gvnpdeavay gaavqgsils geggdetkdi
421 lildvapltl gietvggvmt kliprntvip tkksqvftty qdqqttvsiq vfegersmtk
481 dcrllgkfdl sgipaaprgt pqievtfevd angilnvkae dkgtgkseki titnekgrls
541 qeeidrmvre aeefaeedkk vkeridarnq letyvynmkn tvgdkdklad kleseekekv
601 eealkealew ldenqtaeke eyeeklkeve avcnpiisav yqrtggapgg rrrgrlddeh
661 del
```

EEC69073 / Osi_37938 [Oryza sativa Indica Group] The stromal HSP70 was found to occur in recombinant albumin in Cellastim at about 0.06% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 7**) is provided below:

```
masftsqlga macgaapsts plaarrsgql fvgrkpaaas vqmrvpragr argvamrvac
61 ekvvgidlgt tnsavaameg gkptvitnae ggrttpsvva ytkggerlvg qiakrqavvn
121 pentffsvkr figrkmaevd deakqvsyhv vrddngnvkl dcpaigkqfa aeeisaqvlr
181 klvddaskfl ndkitkavvt vpayfndsqr tatkdagria glevlriine ptaaslaygf
241 ekknnetilv fdlgggtfdv svlevgdgvf evlstsgdth lggddfdkfy fcwvfyfgam
301 thetpkvvdw lasnfkkdeg idllkdkqal qrlteaaeka kmelstlsqt nislpfitat
361 adgpkhiett lsrakfeelc sdlidrlktp vtnalrdakl svdnldevil vggstripsv
421 qelvkkitgk dpnvtvnpde vvslgaavqg gvlagdvkdv vlldvtplsl gletlggvmt
481 kiiprnttlp tsksevfsta adgqtsvein vlqgerefvr dnkslgsfrl dgippaprgv
541 pqievkfdid angilsvaai dkgtgkkqdi titgastlpk devermveea dkfaqedkek
601 rdaidtknqa dsvvyqtekq lkelgdkvpa pvkekvdaki nelkeaiagg stqsmkdama
661 alneevmqig qamynqqpna gaagptpgad agptssggkg pndgdvidad ftdsn
```

Because these proteins only occur at low levels in the new batches of rice recombinant albumin relative to albumin, a pre-requisite for confirming that these contaminants are actually responsible for the superior growth promoting effects of the new batches of recombinant albumin is to determine whether the addition back of these components to albumin restores or enhances the growth promoting activities of the recombinant albumin at levels which are comparable to those actually identified for each component in Cellastim.

Example 9: Separation of heat shock proteins from recombinant albumin by affinity chromatography.

Methods: Cellastim produced using the new process [Lots P0153, P0156, and or P0171] powder was mixed with purified water at approximately 20 g/L. The resulting solution was diafiltered against 50 mM Tris/Cl, pH 7.0 with at least 5 equal volumes of buffer. The resulting solution was passed over an ATP agarose column and the resulting flow through was labeled as fraction A. The column was washed with 5 column volumes of the equilibration buffer and the

material bound to the ATP-agarose was eluted with 50 mM Tris/Cl, 1M KCl, pH7.0. The eluted material was labeled as fraction B. The wash was kept as fraction C. Fraction A was directly concentrated to 100 g/L and diafiltered with d-PBS. Fraction B was concentrated significantly, up to 20 fold or 100 fold in 50 mM Tris/Cl for further analysis. The wash fraction C was kept for further reference. For Western blotting, 10 µg of each protein fraction (by A280, where the e.c. (extinction coefficient) of albumin is 0.53 cm^2/mg and e.c. of Hsp70 is 0.41 cm^2/mg) were loaded on a 4-20% SDS PAGE gel in 2X SDS loading buffer. The samples were heated to 80 °C for approximately 5 minutes before loading. The separation was done at 200V (constant voltage) and ran for approximately 90 minutes. The resulting gel was rinsed in water for 30 minutes to 2 hours and then the proteins were transferred to a Nitrocellulose membrane at 30 mA (constant current) for 2 hours. The resulting blot contained the molecular weight marker proteins as a transfer control and was then blocked in 5% (w/v) milk powder in water. The primary monoclonal antibody (a mouse anti-bovine Hsp70(Sigma/Aldrich #H5147)) was added in 5% milk solution to the blot (1:2500) and the blot was incubated on a rocker with gentle rocking overnight at 4 °C. The blot was then washed 4 times for 10 minutes each in TDN and the secondary antibody (Pierce anti-mouse HRP conjugated) in 5% milk solution which was added at a dilution of 1:2500. After incubation at 4 °C for 2 to 3 hours, the blot was washed 4 times with TDN for 10 minutes each. The resulting blot was then incubated with pico (Pierce) chemiluminescent substrate for 5 minutes. Kodak photographic film was exposed to the blot in a dark room and the subsequent film was developed, rinsed, fixed, rinsed, and dried. To determine accurate transfer of the molecular weight marker position onto the film, a light emitting label was used.

Results: The results are shown in **FIG. 11**. The Western blot pictured shows that the separation scheme produces two populations of proteins in the A (flow through) and B (ATP binding) fractions. The starting material, (lane 2) the fraction A flow through, (lane 3) fraction C wash, (lane 4) and fraction B (lane 5) were tested for the ability to react to the monoclonal antibody. In addition, a commercially available Hsp70 protein that serves as a positive control was loaded in the last lane (lane 10). As shown in the blot in **FIG. 11**, the flow through fraction A (lane 3) does not contain significant amounts of Hsp70. The eluted and concentrated fraction B (lane 4) is highly reactive to the antibody as shown in the blot and indicates at least two distinct bands centered around the 75 kDa molecular weight marker. The wash fraction C (lane 5), indicates the presence of two bands that run at slightly below 75 kDa. In a separate independent experiment, the flow through fraction A (lane 7) again is not reactive to the antibody, and the wash fraction C (lane 8) is also not reactive to the antibody, but the fraction enriched in ATP binding proteins (Fraction B) shown in lane 9 gives the same banding pattern as was seen from the first separation.

Discussion: A separation protocol was developed to separate HSP70 proteins based on their ability to bind to ATP agarose affinity resin, and. was tested for its effectiveness. The procedure involved only minimal sample manipulation, using only ATP agarose, and ultrafiltration to concentrate and conduct buffer changes, and an anti-hsp70 antibody to detect the presence of hsps. The results of the procedure (**FIG. 11**) clearly demonstrates that while in 10 µg of starting material, 10 µg of flow through, or 10 µg of wash fraction there is insufficient hsp70 to be detected by the ant-Hsp antibody. By contrast, the fraction eluted from the ATP agarose column, contains at least two proteins that are clearly recognized by the anti-Hsp70 antibody. The results therefore show that the separation scheme was successful and reproducible on two independent chromatography runs and diafiltrations. Furthermore, the data substantiates the identification of heat shock proteins made by Peptide Mass Finger printing, as discussed earlier, and demonstrates that these proteins are functional and can be readily isolated and enriched by simple ATP agarose chromatography followed with diafiltration. It is concluded that the heat shock proteins co-purify with the recombinant albumin. Such co-purification is consistent with the hypothesis that the heat shock proteins are bound to the albumin, and that the albumin acts to stabilize the heat shock proteins in a stable conformation. Surprisingly, the recombinant albumin / heat shock protein complex retains significant ATPase activity (data not shown) consistent with the presence of function heat shock proteins. This increased activity was further confirmed as providing a growth promoting effect as described below.

Example 10: Impact of the removal of Hsps from rice recombinant albumin on cell viability.

Methods: The separation scheme described in Example 9 was also used to produce fraction A suitable for cell culture testing (**FIG. 12**). The method involves minimal manipulation of fraction A, as it is flowed through an ATP agarose column and then concentrated by diafiltration and buffered with PBS that is suitable for cell culture. The intent of the method is to not introduce new variables into the experiment such that a loss of viability is seen but due to some other reason or cause beyond the removal of ATP binding proteins. Fraction A was tested against the unadulterated control (starting material) for ability to promote hybridoma cell culture viability. The results of the test are shown in **FIG. 12**.

Results: As shown in **FIG. 12** the Cellastim starting material (cross hatched bars), and Part A (solid bars) were tested at the same concentration and compared to the negative control (striped bars). A statistically significant decrease is observable at all four concentrations tested The result indicates that there was a significant loss in the performance of Cellastim after ATP agarose treatment. The treatment resulted in a 28.0, 21.7, 26.7, and 79.5 % loss as compared to Cellastim before removal of ATP binding proteins. In this experiment, care was taken in the

design and handling of the samples to ensure that any inadvertent losses in performance due to sample handling or the accidental introduction of new contaminants were minimized.

Discussion: The cell culture results (**FIG. 12**) demonstrate that it is possible to reduce the performance of the rice derived recombinant albumin by simply passing it over an ATP binding column. This data, when combined with the results shown in Example 9 demonstrates that the depletion of the hsps from albumin by the ATP agarose column directly reduces the cell growth promoting properties of the albumin. This result therefore demonstrates that the superior properties of the albumin arise, at least in part, from the contaminating heat shock proteins in the albumin.

Example 11: Effect of rice derived recombinant human serum albumin in combination with lactoferrin on the proliferation of CHO K1 in serum free culture.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components when added in combination with these components, a series of additional experiments were designed to evaluate and identify optimum combinations of such components.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 μM methotrexate, 50 μg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 μg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5×10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or rLF or various mixtures of the two additives. Control cultures without added rHSA or rLF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. All cultures were grown at 37°C, 6% CO_2 in a humidified environment. The Observed Effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Table E9 shows the number of viable cells after 6 days of culture Cells in media without supplementation proliferated to 3.3 x 10^5 viable cells/ml. Addition of plant derived recombinant human serum albumin (rHSA) or recombinant lactoferrin (rLF), or a combination of the two increased the proliferation of cells as indicated below. Column two shows the Observed Effect of the respective additive(s) above baseline control culture. Column 3 shows the Predicted Effect using a model where the predictive additive effect equals the sum of additive1 and additive2.

TABLE E9				
	Viable cells(e^5)/ml	**Observed Effect**	**Predicted Effect**	**Ratio of Lactoferrin to albumin**
No Additives (Baseline)	3.3	0		
rHSA 0.5 g/L	7.3	4.0		
rHSA 1 g/L	7.9	4.6		
rLF 0.1 g/L	7.5	4.2		
rLF 0.8 g/L	16.0	12.7		
rLF 0.1 g/L +rHSA 0.5 g/L	18.9	15.6	8.2	1 : 5
rLF 0.8 g/L +rHSA 0.5 g/L	29.0	25.7	16.7	1 : 0.625
rLF 0.1g/L +rHSA 1g/L	23.6	20.3	8.8	1 : 10
rLF 0.8g/L +rHSA 1g/L	35.4	**32.1**	17.3	. 1.25

Discussion: Lactoferrin is an essential factor for serum free cell culture media, where it acts to transport iron, additionally Lactoferrin has more recently been identified as a growth factor and inhibitor of apoptosis in some cell types (Huang et al., In vitro Cell. Dev. Biol. Anim. (2008) 44(10) 464-71; Wong et al., Rheumatology 48(1) 39-44; Bi et al., Arch. Immunol. Ther. Exp. (1997)45(4) 315-20,Hashizume et al., Biochem. Biophys. Acta. (1983) 763(4) 377-82). In **Table E9**, the data shows that the addition of 0.1 g/L and 0.8 g/L recombinant lactoferrin resulted in a significant enhancement of cell growth compared to the baseline rate of cell growth consistent with lactoferrins growth stimulating properties.

The growth stimulating effect of lactoferrin was further enhanced by the addition of plant derived recombinant human serum albumin. Unexpectedly the combination of 0.1 g/L lactoferrin and 0.5 g/L plant derived recombinant human serum albumin (1 to five ratio) resulted in a stimulation in cell growth that was approximately 4 fold higher than either albumin alone or lactoferrin alone

tested at the same concentrations. While the combination of 0.1 g/L lactoferrin and 1.0 g/L human serum albumin (1 to 10 ratio) resulted in a stimulation in cell growth that was approximately 4.4 to almost 5 fold higher than either albumin or lactoferrin alone at the same concentrations.

Synergism was also observed when plant derived recombinant human serum albumin was added to the lactoferrin at a concentration of 0.8 g/L. Even at this significantly higher concentration, the addition of plant derived recombinant human serum albumin further increased cell growth by 2 to 2.5 fold

Albumin and lactoferrin are distinct protein families with little overlapping structural or functional similarity. Thus the fact the synergism was observed with these proteins is unexpected based on their dissimilar structure & function. Synergism provides the opportunity to develop improved supplements that provide for supplements with enhanced cell culturing properties (i.e. improved cell growth and viability) by using optimal ratios of lactoferrin and albumin.

Example 12: Effect of rice derived recombinant albumin in combination with lactoferrin on the productivity of CHO K1 cells grown in serum free culture

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of plant derived recombinant human serum albumin or LF or various mixtures of the two additives. Control cultures without added HSA or LF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. Antibody productivity was determined at the end of the batch culture when cell viability had declined to <10% (typically day 13 or 14) by quantitative ELISA (Bethyl Laboratories). All cultures were grown at 37°C, 6% CO_2, in a humidified environment. The

Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

TABLE E10				
	Product Conc. (µg/ml)	**Observed Effect**	**Predicted Effect**	**Ratio of HSA to Lactoferrin**
No Additives (Baseline)	47.1	0		
rHSA 0.5g/L	114.6	67.5		
rHSA 1g/L	136.1	89.0		
rLF 0.1g/L	91.9	44.8		
rLF 0.8 g/L	138.5	91.4		
rLF 0.1g/L + rHSA 0.5g/L	183.7	136.6	112.3	1 : 5
rLF 0.8g/L + rHSA 0.5g/L	242.1	195.0	158.9	1 : 0.625
rLF 0.1g/L + rHSA 1g/L	219.3	172.2	133 8	1 : 10
rLF 0.8g/L + rHSA 1g/L	260.2	213.1	180 4	1. 1 25

Table E10 shows the effect of plant derived recombinant human serum albumin and recombinant lactoferrin produced in monocot cells, and various combinations of the two proteins on the productivity of CHO K1 cells. Table E10 shows that the concentration of antibody product produced by the cells after 13 days of culture in the baseline medium (with no additives) produced 47.1 µg/ml of antibody product. Medium supplemented with recombinant albumin or recombinant lactoferrin alone or a combination of plant derived recombinant human serum albumin and lactoferrin produced significantly higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the predictive additive effect of the combination of two additives using a model where the predictive additive equals the sum of each additive alone at the same concentration.

Discussion: Table E10 demonstrates that improvements in cell growth and viability directly translated into improved antibody expression and cell productivity. As was the case for cell viability and cell growth, maximal effects of the protein components was observed when these were added to the culture together.

Table E11 shows an additional data set showing the effect of the additives at an expanded range of concentrations, using the same conditions as in **Example12.** Medium supplemented with recombinant albumin alone, or lactoferrin alone, or a combination of both proteins, produced higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the Predicted Effect of the combination of two additives using a model where the Predicted Effect equals the sum of the effects of each additive when added alone at the same concentration. The data indicates that the actual effect of the combination is greater than the Predicted Effect over all of the ratios of the two proteins tested.

TABLE E11

	Product Conc. (ug/ml)	Observed Effect	Predicted Effect	% Improvement over predicted activity	Ratio of Lactoferrin to HSA
No additives (Baseline)	56.9	0.0			
Single additives					
HSA 0.5g/L	99.1	42 2			
HSA 1 g/L	126.3	69.4			
HSA 2 g/L	186.0	129.1			
HSA 3 g/L	212.9	156.0			
LF 0.1g/L	123.1	66.2			
LF 0.5g/L	173.5	116.6			
LF 0.8g/L	182.0	125.1			
LF 1.5g/L	196.3	139.4			
Combinations					
LF 0.1g/L+ HSA 0.5g/L	212.4	155.5	108.5	143%	1 : 5
LF 0.5g/L+ HSA 0.5g/L	314.9	258.0	158.9	163%	1: 1
LF 0.8g/L+ HSA 0.5g/L	302 8	245.9	167.4	147%	1: 0 625
LF 1.5g/L+ HSA 0.5g/L	336 8	279.9	181.7	154%	1: 0 33

LF 0.1g/L+ HSA 1.0g/L	239 7	182.8	135.7	133	1: 10
LF 0.5g/L+ HSA 1.0g/L	339 4	282.5	186.1	152	1: 2
LF 0.8g/L+ HSA 1 0g/L	357.7	300.8	194.6	155	1: 1.2
LF 1 5g/L+ HSA 1 0g/L	343.7	286.8	208.9	137	1: 0.66
LF 0.1g/L+ HSA 2.0g/L	261.3	204.4	195.4	105	1: 20
LF 0.5g/L+ HSA 2.0g/L	377 2	320.3	245.8	131	1: 4
LF 0.8g/L+ HSA 2.0g/L	414 8	357.9	254.3	141	1: 2.4
LF 1.5g/L+ HSA 2.0g/L	358.0	301.1	268.6	112	1: 1.33

Discussion: Ratios of lactoferrin to albumin as low as 1 to 0.333 to as high as 1: 20 produced synergistic stimulations of cell growth. Optimal ratios of lactoferrin to albumin were found at 1 to 0.333 wt/wt (at an albumin concentration of 0.5 g/L), at 1 to 1.2 wt/wt (at an albumin concentration of 1.0 g/L); and at 1: 2.4 wt/wt (at an albumin concentration of 2.0 g/L).

Thus the stimulation of cell productivity occurs over a broad range of ratios of recombinant albumin and lactoferrin. Optimal ratios of lactoferrin and recombinant albumin vary to a certain extend on the absolute concentration of both protein components. Preferred ratios of Lactoferrin to Albumin range from about 1 (lactoferrin) to 0.33(albumin) to about 1 (lactoferrin) to 3 (albumin) wt/wt.

Example 13: Evaluation of the effect of combinations of albumin and transferrin in the presence of other cell culture components.

To determine the effect of other cell culture components on the synergistic effect of lactoferrin and albumin on cell growth and viability, a series of additional experiments were carried out with other cell culture components which have been previously shown to be essential for cell growth in serum free conditions.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments transferrin (TF) was used at an initial concentration of 5.5 µg/mL; Plant derived recombinant

Human Serum Albumin (HSA) was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite (SE) and Ethanolamine were used at concentrations of 1:500,000 v/v and 6 7 µg/mL respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the supplements listed above, as described in **Table E12** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C. 6% CO_2, in a humidified environment.

TABLE E12

	Viable cells/ ml	Std	Observed Effect	Predicted Effect	Synergistic	Ratio of transferrin to albumin
DMEM/F12 alone	0.01	0.01				
10% FBS	11.40	0 71	11.39			
TF 5.5 µg/L	0.02	0.00	0.01			
SE 6.7 µg/L	0.22	0.13	0.21			
HSA 0.1 g/L	2.31	0.06	2.30			
HSA 0.5 g/L	4.67	0.18	4.66			
HSA 1 g/L	4.55	0.46	4.54			
doubles						
TF+SE	0.40	0.04	0.38	0.21	yes	
TF+ HSA 0.1 g/L	3.00	0.02	2.98	2.30	yes	1:18
TF+ HSA 0.5 g/l	5.42	0.20	5.41	4.67	yes	1:90
TF+ HSA 1 g/L	5.71	0.14	5.69	4.55	yes	1:181
doubles						
SE+ HSA 0.1 g/l	2.76	0 06	2.75	2.50	Yes/no	
SE+ HSA 0.5 g/L	4.15	0.16	4.14	4.87	no	

SE+ HSA 1 g/L	3.75	0.22	3.74	4.75	no	
Triples						
TF+SE+ HSA 0.1 g/L	7.40	0.00	7.39	2 51	yes	*1:18*
TF+SE+ HSA 0.5 g/L	9.48	0.31	9.46	4.87	yes	*1:90*
TF+SE+ HSA 1 g/L	9.71	0.11	9.70	4.75	yes	*1 181*

Discussion: Recombinant human serum albumin in combination with recombinant transferrin showed synergistic effects when added to culture media together. This effect was further augmented in the presence of selenium. However the addition of selenium did not act synergistically with recombinant plant derived human serum albumin alone. Maximal synergistic effects of transferrin and plant derived recombinant human serum albumin occurred in the presence of selenium and at ratios of transferrin to plant derived recombinant human serum albumin of about 1 (transferrin) to 100 (albumin) wt/wt. and of about 1 (transferrin) to 200 (albumin).

Selenium is a well known essential trace element for cell growth and development, and its positive role in biological systems includes detoxification of free radicals by activating glutathione peroxidase. Additionally selenium can act as a highly effective iron carrier in serum free media, which helps to explain its synergistic impact on transferrin stimulated cell growth. (Zhang et al., Biotechnol. Bioeng. (2006) 95(6) 1188-97)

Example 14: Evaluation of the effect of combinations of albumin, transferrin and insulin.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin, plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination with insulin was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 0.1 g/L, 0.5 g/L, and 1 g/L & Sodium Selenite and Ethanolamine were used at concentrations of 6.7 µg/mL and 1:500,000 v/v and respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E13** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment, and shaken with 2 cm rotational orbit at 120 RPM.

TABLE E13

	Viable cells/ml	Std	Observed Effect	Predicted Effect	Synergistic
D/F12 alone	0.01	0.01			
10% FBS	11.65	0.21	11.64		
Factors Alone					
Insulin (IN) 10 µg/L	0.04	0.02	0.03		
Transferrin (TF) 5.5 µg/L	0.02	0.00	0.01		
Sodium Selenite (SE) 6.7 µg/L	0.22	0.13	0.21		
HSA 0.1 g/L	2.31	0.06	2.30		
HSA 0.5 g/L	4.67	0.18	4.66		
HSA 1 g/L	4.55	0.46	4.54		
Doubles					
IN+TF	0.03	0.02	0.02	0.06	
IN+SE	1.06	0.15	1.05	0.26	Yes
IN+ HSA 0.1 g/L	2.54	0.08	2.53	2.35	No
IN+ HSA 0.5 g/L	3.96	0.34	3.95	4.71	No
IN+ HSA 1 g/L	4.61	0.16	4.60	4.59	No
Triples					
IN+TF+SE	2.13	0.49	2.12	0.28	Yes
IN+TF+ HSA 0.1 g/L	3.29	0.03	3.28	2.37	Yes
IN+TF+ HSA 0.5 g/L	5.84	0.49	5.83	4.73	Yes
IN+TF+ HSA 1 g/L	6.84	0.34	6.83	4.61	Yes

Triples					
IN+SE+ HSA 0.1 g/L	2.21	0.17	2.19	2.37	No
IN+SE+ HSA 0 5 g/L	3.41	0.21	3.39	4.73	No
IN+SE+ HSA 1 g/L	3 62	0.23	3.60	4.61	No
Quaternaries					
IN+TF+SE+ HSA 0 1 g/L	7.91	0.46	7 90	2 59	Yes
IN+TF+SE+ HSA 0 5 g/L	9.14	0.63	9 13	4 95	Yes
IN+TF+SE+ HSA 1 g/L	10.07	0.67	10.05	4.83	Yes

Discussion: In these studies the addition of insulin and plant derived recombinant human serum albumin did not produce a synergistic effect on cell growth. By contrast the addition of plant derived recombinant human serum albumin and transferrin and insulin did produce a synergistic effect on cell growth.

Example 15: Evaluation of the effect of combinations of albumin, transferrin and insulin in the presence of Selenium.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin,(HSA) insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 6 7 µg/m L Sodium Selenite at 0.4x 10^5 viable cells/mL In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL Ethanolamine were used at a concentration of 1·500,000 v/v The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E14** below. Control cultures without added supplements were used as the baseline All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E14						
Condition	Viable cell count 10e5	Std	Observed Effect	Predicted Effect	% Improvement over predicted activity	Synergistic
Base medium of D/F12+ Selenium	0.08	0.04	0.00			
10% FBS	13.20	0 28	13.12			
Insulin	0.30	0.09	0.22			
Factors Alone						
TF	0.27	0.04	0.19			
HSA 0.1 g/L	2.23	0.11	2.15			
HSA 0.5 g/L	6.21	0.20	6 13			
HSA 1 g/L	8.00	0 05	7 91			
doubles						
IN+TF	0.48	0.02	0.40	0.41		No
IN+ HSA 0.1 g/L	1.81	0.02	1.72	2.37		No
IN+ HSA 0.5 g/L	6.00	0.09	5.91	6.35		No
IN+ HSA 1 g/L	7.43	0 19	7.35	8.13		No
doubles						
TF+ HSA 0 1 g/L	4.29	0.04	4.20	2.34	179	Yes
TF+ HSA 0 5 g/L	12.35	0.21	12.27	6.32	194	Yes
TF+ HSA 1 g/L	15.48	0.71	15.39	8.1	190	Yes
Triples						
IN+TF+ HSA 0.1 g/L	4.18	0.21	4.09	2.56	159	Yes
IN+TF+ HSA 0.5 g/L	13.60	0.00	13.52	6.54	206	Yes
IN+TF+ HSA 1 g/L	15.38	0 22	15.29	8.32	184	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin.

Example 16: Evaluation of the effect of combinations of albumin and transferrin in the presence of Ethanolamine.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin (HSA), insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 1:500,000 Ethanolamine v/v at 0.4x 10^5 viable cells/ml. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite was used at a concentration of 6.7 µg/mL. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E15** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E15				
Condition	Observed Effect (Viable cells/ml)	Std	Predicted Effect (Viable cells/ml)	Synergistic
Base Medium of D/F12+ Ethanolamine	0.51	0.16		
10% FBS	14.45	0.64		
IN	1.41	0.24		
Factors Alone				
TF	2.08	0.15		
HSA 0.1 g/L	2.16	0.07		
HSA 0.5 g/L	5.66	0.35		
HSA 1 g/L	7.08	0.21		
doubles				
IN+TF	3.12	0.11	3.48	No
IN+ HSA 0.1 g/L	1.87	0.00	3.57	No
IN+ HSA 0.5 g/L	4.48	0.25	7.07	No
IN+ HSA 1 g/L	5.14	0.19	8.49	No
doubles				
TF+ HSA 0.1 g/L	6.72	0.35	4.24	Yes
TF+ HSA 0.5 g/L	12.30	0.26	7.74	Yes
TF+ HSA 1 g/L	16.20	0.65	9.16	Yes
Triples				
IN+TF+ HSA 0.1 g/L	7.46	0.28	5.64	Yes
IN+TF+ HSA 0.5 g/L	13.30	0.36	9.15	Yes
IN+TF+ HSA 1 g/L	16.60	0.48	10.57	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin, over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin under these conditions.

Surprisingly the combination of Insulin, transferrin and plant derived recombinant human serum albumin produced superior cell growth compared to 10 % serum, demonstrating the superior properties of these combinations of proteins.

Example 17: Comparison of rice recombinantly produced albumin and plasma derived albumin.

To directly compare the effect of rice derived recombinant human serum albumin (rHSA) to plasma derived serum albumin (pHSA), samples of both were directly compared at two ratios when mixed with rice derived recombinant transferrin for their ability to stimulate cell growth.

Methods: Hybridoma cells were grown in DMEM/F12 (Gibco) in 10% FBS. Cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in either 1)DMEM/F12 base medium, 2) DMEM/F12+sodium selenate base medium or 3) DMEM/F12 base medium at $0.75x10^5$ viable cells/ml. Each base medium was further supplemented with either recombinant transferrin (TF), rice derived recombinant human serum albumin (rHSA), plasma derived human serum albumin (pHSA) or combinations of both transferrin and either form of albumin. After 3 days of incubation at 37° C, 5% CO_2, the concentration of viable cells was determined by a Guava PCA cell counter as instructed by the manufacturer. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components. The Effect Factor was calculated as the observed effect divided by the expected effect. Effect factor of 1 is indicative of expected effect (additive). Effect factor >1 is indicative of more than additive or synergistic effect.

In these experiments transferrin (TF) was used at an initial concentrations of 1 and 3 mg/L; albumins were used at concentrations of 5 g/L, Sodium Selenite was used at a concentration of

6.7 µg/L & Ethanolamine was used at 1:500,000 v/v.

Table E16					
Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual / expected
Base Medium	0.11	0.07			
Components alone					
rTF 1 mg/L	0.21	0.01	0.10		
rTF 3 mg/L	0.28	0.09	0.18		
pHSA 5 g/L (Seracare)	11.30	0.28	11.19		
rHSA 5 g/L (Cellastim)	5.11	0.02	5.00		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	12.85	0.49	12.74	11.51	1.1
rTF3 mg/L + pHSA 5 g/L	13.20	0.42	13.09	16.41	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	18.80	0.71	18.69	5.31	3.5
rTF3 mg/L + rHSA 5 g/L	18.85	0.92	18.74	5.39	3.5

Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual/expected
Base Medium	0.04	0.02			
Components alone					
rTF 1 mg/L	0.14	0.01	0.10		
rTF 3 mg/L	0.14	0.05	0.10		
pHSA 5 g/L	6.08	0.13	6.04		
rHSA 5 g/L	1.76	0.17	1.72		

pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	7.46	0.49	7.42	6.22	1.2
rTF3 mg/L + pHSA 5 g/L	7.84	0.05	7.79	7.84	1.0
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	8.55	0.79	8.51	1.90	4.5
rTF3 mg/L + rHSA 5 g/L	8.37	0.23	8.33	1.90	4.4

Base Medium:DMEM/F12	**Viable cells/ml 10e5**	**Std**	**Observed Effect**	**Predicted Effect**	**Effect Factor (actual/ expected**
Base Medium	1.01	0.02			
Components alone					
rTF 1 mg/L	3.45	0.27	2.44		
rTF 3 mg/L	3.47	0.27	2.46		
pHSA 5 g/L	13.15	0.49	12.14		
rHSA 5 g/L	3.46	0.13	2.45		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	14.83	0.49	13.82	16.60	0.8
rTF3 mg/L + pHSA 5 g/L	14.57	0.12	13.56	16.61	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	17.87	1.46	16.86	6.91	2.4
rTF3 mg/L + rHSA 5 g/L	20.20	1.13	19.19	6.93	2.8

Discussion: Table E14 shows the results of three independent experiments that confirm that the combinations of TF with rice derived recombinant human serum albumin produced Observed Effect values much greater than the Predicted Effect. By comparison, the combination of ***plasma***-derived HSA and transferrin produced values that were similar to, or less than, the expected effect. Thus the ability of albumin to synergistically enhance the activity of transferrin

and lactoferrin appears to be dependent on the source of albumin, rather than being an inherent characteristic of the albumin itself Specifically the present experiments establish that the synergistic properties of human serum albumin with transferrin related proteins such as transferrin and lactoferrin is an unexpected and surprising property of rice derived recombinant human serum albumin. Significantly the combination of transferrin and plant derived recombinant albumin was more effective in stimulating cell growth than the combination of plasma derived albumin and transferrin, even though plasma derived albumin was more effective at 5 g/L alone than the same concentration of rice derived human serum albumin.

Example 18: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with either plasma derived or recombinant transferrin.

To directly compare the effect of rice derived recombinant human transferrin (rTF) to plasma derived transferrin (pTF), samples of both were directly compared when mixed with rice derived recombinant human serum albumin for their ability to stimulate cell growth.

Methods: A base medium (BM) was composed that consisted of DMEM/F12+ with Sodium Selenite 0.0067 mg/L, Ethanolamine 1:500,000 v/v. Base medium included human plasma transferrin, pTF at a concentration of 5.5 mg/L in combination with either plasma HSA (pHSA, cell culture grade Seracare) or rice derived recombinant HSA (rHSA). Hybridoma cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in at $0.80x10^5$ viable cells/ml and incubated at 37° C, 6% CO_2, The concentration of viable cells was determined daily by a Guava PCA cell counter as instructed by the manufacturer. The concentration of antibody produced by the cells and secreted into the medium was determined on day 6 by quantitative ELISA (Bethyl laboratories) Error bars indicate standard deviation The production of antibody with the combination of pTF +rHSA was similar to that of DMEM/F12+10% FBS.

Discussion: FIG. 13 shows that rice derived recombinant human serum albumin was able to act synergistically with plasma derived transferrin to significantly enhance the growth and productivity of the cells. By comparison, plasma derived serum albumin was unable to exert a similar stimulatory effect. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective with transferrin related proteins that were either plasma derived, or recombinant.

Example 19: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with insulin related growth factor-1.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components such as the growth factor IGF-1, additional experiments were performed to assess this possibility.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) as described previously. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and recombinant insulin related growth factor 1 (IGF1) (Ajinomoto) alone or in combination were examined Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1 5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or IGF-1 or various mixtures of the two additives. Control cultures without added rHSA or IGF-1 were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture All cultures were grown at 37°C, 6% CO_2, in a humidified environment. Table E17 shows the results of the experiment. Here the Observed Effect was calculated as the net viable cell concentration (observed-base medium), and the Predictive Effect was calculated as the sum of the effect of the individual components.

Table E17

	viable cells/ml 10e5	Observed Effect (Cell count-baseline)	Predicted Effect
No Additive(baseline)	3 3	0.0	
HSA 0.5 g/L	7.3	4 0	
HSA 1g/L	7.9	4.6	
IGF-1 30 nM	12.0	8.7	

HSA 0.5 g/L+ IGF-1 30nM	23.5	15.6	12.7
HSA 1 g/L+ IGF-1 30nM	25.6	13.6	13.3

Results and Discussion: **Table E17** shows that rice derived recombinant human serum albumin was able to act synergistically with recombinant IGF-1 to significantly enhance the growth and productivity of the cells. By comparison, recombinant albumin did not act synergistically with insulin (Table E15) suggesting that synergism is not a general effect across all cell culture components. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective in combination with IGF-1.

It will, of course, be appreciated that the above description has been given by way of example only and that modifications in detail may be made within the scope of the present invention.

The invention is capable of considerable modification, alteration, and equivalents in form and function, as will occur to those ordinarily skilled in the pertinent arts having the benefit of this disclosure.

While the present invention has been described for what are presently considered the preferred embodiments, the invention is not so limited. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the detailed description provided above.

ABSTRACT OF THE DISCLOSURE

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

Cell Culture Media Containing Combinations of Proteins

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Serial No. 12/798,462, filed February 18, 2010, which claims the benefit of US provisional patent application No. 61,154,204, filed February 20, 2009, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

BACKGROUND OF THE INVENTION

Cell culture techniques allow animal or plant cells that are removed from tissues to grow when supplied with the appropriate nutrients and conditions. The cells are capable of dividing and can continue to grow until limited by culture variables such as nutrient depletion or toxic buildup (Butler, M. & Jenkins, H., "Nutritional aspects of growth of animal cells in culture," J of Biotechnol. (1989) 12: 97-110). Cell culture techniques have a number of applications including investigation of the normal physiology or biochemistry of cells (Balaban, B. & Urman, B., "Embryo culture as a diagnostic tool," Reprod. Biomed. Online (2003) 7(6): 671-82), testing the effect of various chemical compounds or drugs on specific cell types (Farkas, D. & Tannenbaum, S.R., "In vitro methods to study chemically-induced hepatotoxicity: a literature review," Curr. Drug Metab. (2005) 6(2): 111-25), studying the sequential or parallel combination of various cell types to generate artificial tissues (Wang et al., "Cartilage tissue engineering with silk scaffolds and human articular chondrocytes," Biomaterials (2006)), and synthesizing valuable biologics from large scale cell cultures (Zeilinger et al., "Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions," Altern. Lab Anim. (2002) 30(5): 525-38). Cell culture techniques have also been used for in vitro fertilization (Blake et al., "Protein supplementation of human IVF culture media," J Assist Reprod. Genet. (2002) 19(3): 137-43; Bungum et al., "Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study," Reprod. Biomed. Online (2002) 4(3): 233-6), stem cell research (Conley et al., "Derivation, propagation and differentiation of human embryonic stem cells," Int. J Biochem Cell Biol. (2004) 36(4): 555-67), vaccine production (Chuang et al., "Pharmaceutical strategies utilizing recombinant human albumin," Pharm. Res. (2002) 19(5): 569-77; GlaxoSmithKline, HAVRIX® (Hepatitis A Vaccine, Inactivated) - Prescribing Information

(2005), available at http://us.gsk.com/products/assets/us_havrix.pdf; Innis et al., "Protection against hepatitis A by an inactivated vaccine," JAMA (1994) 271(17): 1328-34, Merck, PROQUAD® - Measles, Mumps, Rubella, and Varicella (Oka/Merck) Virus Vaccine Live - Prescribing Information (2005), available at http://www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf; Litwin, J., "The growth of Vero cells in suspension as cell-aggregates in serum-free media," Cytotechnology (1992) 10(2): 169-74), tissue engineering including artificial skin (Atala, A., "Future perspectives in reconstructive surgery using tissue engineering," Urol. Clin North Am. (1999) 26(1): 157-65, ix-x; Sher, et al., "Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation," J Biol. Chem. (2006) 281(8): 5178-87) and organs (Neronov et al., "Integrity of endothelium in cryopreserved human cornea," Cryo Letters (2005) 26(2): 131-6; Han, et al., "Interleukin-1 alpha-induced proteolytic activation of metalloproteinase-9 by human skin," Surgery (2005) 138(5): 932-9) and gene and cell therapy (Chadd, H E. & Chamow, S.M., "Therapeutic antibody expression technology," Curr. Opin. Biotechnol. (2001) 12(2): 188-94).

Biologics encompass a broad range of cell products, and include specific proteins or viruses that require animal cells for propagation. For example, therapeutic proteins such as monoclonal antibodies can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures (Dewar et al., "Industrial implementation of in vitro production of monoclonal antibodies, Ilar J (2005) 46(3): 307-13). The number of such commercially valuable biologics has increased rapidly over the last decade and has led to the present widespread interest in mammalian cell culture technology (Mizrahi, A., "Biologicals produced from animal cells in culture – an overview," Biotechnol. Adv. (1988) 6(2). 207-20).

The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The need for cell culture, especially at large scale, became apparent with the need for viral vaccines. Major epidemics of polio in the 1940s and 1950s promoted efforts to develop an effective vaccine. In 1949, it was shown that poliovirus could be grown in cultures of human cells, which led to considerable interest in the development of large quantities of the polio vaccine using cell culture (Ligon, B.L., "Thomas Huckle Weller MD: Nobel Laureate and research pioneer in poliomyelitis, Varicella-zoster virus, cytomegalovirus, rubella, and other infectious diseases," Semin. Pediatr. Infect. Dis. (2002) 13(1): 55-63) The polio vaccine, produced from de-activated virus, became one of the first commercial products of cultured animal cells (Furesz, J., "Developments in the production and quality control of poliovirus vaccines – Historical perspectives," Biologicals (2006))

Due to the safety and ethical considerations associated with the use of animal-derived cell culture media components, efforts have been made to provide alternative sources for cell culture media and media components

Published PCT Appl. No. WO 2007/002762, which was based on U.S. Provisional Appl. No. 60/694,236, filed June 28, 2006, relates to recombinant production of components of cell culture media using plant cells, and cell culture media containing such recombinant proteins. The entire contents of this application are incorporated herein by reference.

SUMMARY OF THE INVENTION

Accordingly, it is an objective of the invention to provide cell culture media or media supplements containing combinations of protein components, which when used in cell culture, result in improved growth and/or productivity of cultured cells.

One embodiment of the invention is a cell culture media including a cell culture media base, and two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

Yet another embodiment of the invention is a cell culture media including a cell culture media base, albumin, and at least one additional protein selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the growth factors that may be provided in the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

Another embodiment of the invention is a cell culture media including a cell culture media base, lactoferrin, and at least one additional protein selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to further embodiments, a preferred combination of proteins that may be provided in the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides methods for producing supplemented cell culture media, including providing a cell culture media base; providing two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin; and combining the two or more proteins with the cell culture media base to form the supplemented cell culture media.

According to further embodiments, the proteins used in the methods for producing cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be provided in the methods of producing the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of producing the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides a method for culturing cells, including providing a cell culture media base, supplementing the cell culture media base with two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin to form a supplemented cell culture media, and introducing cells to be cultured into the supplemented cell culture media. The cells grown in the supplemented cell culture media exhibit improved growth characteristics as compared to cells grown in an unsupplemented cell culture media.

According to further embodiments, the proteins used in the methods for culturing cells are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for culturing cells are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be used in the methods for culturing cells are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, by culturing the cultured cell in the cell culture media of the present invention

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100,000, to about 1 to 1,000.

In one aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein. In aspect of this embodiment, the heat shock protein is a rice heat shock protein. In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein. In another aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469

In another aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt/wt HSP70.

In another aspect of any of the claimed supplements, the transferrin related protein is lactoferrin. In another aspect of any of the claimed supplements, the transferrin related protein is transferrin. In one aspect, of any of the claimed supplements the lactoferrin is human. In one aspect, of any of the claimed supplements the transferrin is human. In one aspect, of any of the claimed supplements the lactoferrin is recombinant. In one aspect, of any of the claimed supplements the lactoferrin is derived from plasma. In one aspect, of any of the claimed supplements the transferrin is recombinant. In one aspect, of any of the claimed supplements the transferrin is derived from plasma. In one aspect, of any of the claimed supplements the IGF-1 is human. In one aspect, of any of the claimed supplements the IGF-1 is recombinant.

In another aspect of any of the claimed supplements, the ratio of transferrin to albumin about 1 to 50, to about 1 to 200. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 75, to about 1 to 180 In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 80, to about 1 to 120. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 1000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 2000. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 3000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 4000. in another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 5000.

In another aspect of any of the claimed supplements. the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0 5, to about 1 to 2 In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and a

transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises. a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein. wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of

albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin. and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the cell culture medium wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a

plant. ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated

bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 5000. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In one aspect of any of these methods, the supplement further comprises insulin. In one aspect of any of these methods, the supplement further comprises IGF-1. In another aspect, the supplement further comprises selenium and ethanolamine.

In another aspect of any of the claimed methods, the cells are tissue culture cells. In another aspect, the cells are CHO cells. In another aspect the cells are hybridoma cells. In another aspect the cells are vero cells. In another aspect the cells are sorted by flow cytometry. In another aspect the cells are primary cells. In one aspect, the primary cells are embryonic stem cells. In another aspect the primary cells are B-cell derived. In another aspect the cells the primary cells are T-cell derived. In another aspect the cells are B-cells or B-cell derived. In another aspect the cells are T-cells or T-cell derived. In another aspect the cells. In another aspect the cells are isolated by a micro fluidic device. In another aspect the cells are isolated by single-cell subcloning

In one aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt / wt of a heat shock protein In aspect of this embodiment, the heat shock protein is a rice heat shock protein In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein In another

aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469.

In another aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt /wt HSP70

In another aspect of any of the claimed methods, the transferrin related protein is lactoferrin. In another aspect of any of the claimed methods the transferrin related protein is transferrin. In one aspect, of any of the claimed methods the lactoferrin is human. In one aspect, of any of the claimed methods the transferrin is human. In one aspect, of any of the claimed methods the lactoferrin is recombinant. In one aspect, of any of the claimed methods the transferrin is recombinant.

In another aspect of any of the claimed methods, the ratio of transferrin to albumin is about 1 to 50, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 75, to about 1 to 180. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 80, to about 1 to 120. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 120, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 200, to about 1 to 1000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 1000, to about 1 to 2000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 2000, to about 1 to 3000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 3000, to about 1 to 4000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 4000, to about 1 to 5000.

In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.5, to about 1 to 2. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 100 mg /L and 400 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 400 mg /L and 800 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 800 mg /L and 1000mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 1000 mg /L and 2000 mg/ L.

In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 1ug /L and 50 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 2 ug /L and 10 ug/ L In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 3 ug /L and 8 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 4 ug /L and 6 ug/ L.

In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 100 mg /L and 2000 mg/ L. In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 200 mg /L and 1000 mg/ L.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 30 % compared to the summed effect of recombinant albumin and transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 40 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 50 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 60 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 70 % compared to the summed effect of recombinant albumin or transferrin related protein alone when measured under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 80 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 90 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 100 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 150 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions

In another aspect of any of the claimed methods the improvement in cell viability is greater than 200 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

Other novel features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

BRIEF DESCRIPTION OF THE FIGURES

For a more complete understanding of the present invention, the needs satisfied thereby, and the objects, features, and advantages thereof, reference now is made to the following description taken in connection with the accompanying drawings.

~~Figure~~FIG. 1A is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1B is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes

FIG. 1C is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1D is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

~~Figure~~FIG. 2 is a plasmid map of the 5,817 bp plasmid, API164 (GT1-Lac), showing an expression cassette for human lactoferrin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human lactoferrin, a Nos terminator and a kanamycin resistance selectable marker.

~~Figure~~FIG. 3 shows the results of a SDS-PAGE analysis for the expression of recombinant human lactoferrin. Total proteins from rice seed extracts were suspended in Laemli sample buffer, run on a gradient gel and stained with Coomassie blue. Lane 1 is the molecular weight marker. Lanes 3 to 6 are purified human derived lactoferrin (Sigma Chemical, USA). Lanes 8 to 13 are single seed extracts from homozygous independent transgenic rice lines and lane 14 is a seed extract from non-transformed rice variety Taipei 309

~~Figure~~FIG. 4 is a stable expression of recombinant human lactoferrin in transgenic rice grains from R_2 through R_{10} generations. Total soluble proteins from 1 g of brown rice flour was extracted with 40 ml of extraction buffer and clarified by centrifugation. The extract was analyzed via ELISA. Extraction was repeated three times and standard deviation is shown as an error bar.

~~Figure~~FIG. 5A shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5B shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein

sequence derived from P02768 (Swiss-Prot).

FIG. 5C shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5D shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

~~Figure~~FIG. 6 is a plasmid map of the 5,496 bp plasmid, API504 (GT1-HSA), showing an expression cassette for human albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

~~Figure~~FIG. 7 is a plasmid map of the 12,388 bp plasmid, API508 (JH/GT1-HSA), showing an expression cassette for human serum albumin (HSA) and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

~~Figure~~FIG. 8A-FIG. 8E Shows a comparison by HPLC size exclusion chromatography of recombinant HSA produced from rice compared to other sources of albumin and methods of purification. ~~Figure~~FIG. 8A shows the chromatogram for a serum derived (non-recombinant HSA). ~~Figure~~FIG. 8B shows the chromatogram for a rice recombinant HSA (Cellastim P0107) made using the "old process" B000 for purification. ~~Figure~~FIG. 8C shows the chromatogram for a rice recombinant HSA (Cellastim P0171) made using the "new process" B0000C for purification. ~~Figure~~FIG. 8D shows an overlay of the chromatograms for the serum derived albumin (1A; dotted line) and Cellastim HSA prepared using the new process ((1C; solid line). ~~Figure~~FIG. 8E shows an overlay of the chromatograms for HSA prepared using the old process B000 (Cellastim P0107) (1B; dotted line) and HSA prepared using the new process B0000C (Cellastim P0171) (1C; solid line).

~~Figure~~FIG. 9A-FIG. 9B Shows a comparison by SDS PAGE analysis of recombinant albumin produced from rice compared to other sources of HSA and methods of purification. ~~Figure~~FIG. 9A shows a comparison of Cellastim P0171albumin and Celiprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin

(10 µg) and Lane 7 is the Celiprime albumin (10 µg). ~~Figure~~FIG. 9B shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8) The six samples were loaded at 20 µg per lane.

~~Figure~~FIG. 10A: Shows a comparison of the effects of yeast recombinant HSA (Celiprime), human plasma derived HSA, (Seracare) and plant recombinant HSA (Cellastim P0171) with respect to their ability to promote cell growth and viability. ~~(Figure 10A).~~ ~~Figure~~FIG. 10B shows a comparison of the endotoxin levels in batches of albumin produced using the old (B000) and new processes (B0000C) for recombinant albumin production. ~~Figure~~FIG. 10C shows a comparison of cell growth and viability of cells grown in the presence of the Cellastim HSA produced using the old (B000) and new processes (B0000C) for recombinant albumin production.

~~Figure~~FIG. 11: Shows a western blot of two independent experiments showing that plant derived recombinant HSA comprises proteins that can be selectively concentrated by an ATP affinity column (Lanes 4 & 9) and react against an anti-HSP antibody. Lanes 3 and 7 show 10 ug of starting material; Lanes 4 and 9 show the fractions after elution and concentration from the ATP affinity column; Lanes 5 and 8 show the wash through after elution with ATP, and lane 10 is a hsp positive control. (See Example 9 for details).

~~Figure~~FIG. 12: Shows a comparison of the cell growth and viability effect of Cellastim recombinant albumin after passing the albumin produced using the new process over an ATP affinity column to remove heat shock proteins. (See text for details).

~~Figure~~FIG. 13: Shows a comparison of the effect of 1 g/L recombinant plant derived human serum albumin (rHSA) in combination with transferrin (pTF) compared to the same concentration of plasma derived human serum albumin (pHSA) in combination with transferrin (pTF) on the production of antibody by hybridoma cells in culture.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description. Unless otherwise indicated, all terms used herein have the meanings given below, or are generally consistent with same meaning that the terms have to those skilled in the art of the present

invention. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al (1993), *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y., and Gelvin and Schilperoot, eds (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands, for definitions and terms of the art.

The term "**about**" or "**approximately**" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviations, per practice in the art. Alternatively, "about" with respect to the compositions can mean plus or minus a range of up to 20%, preferably up to 10%, more preferably up to 5%.

The term "**antigen-binding fragment**" refers to a polypeptide portion of an immunoglobulin or antibody that binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). Binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies.

The term "**apoptosis**" ("normal" or "programmed" cell death) refers to the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine dependent tissue atrophy. Apoptosis may also be triggered in cells grown under tissue culture conditions in response to stress. Cells undergoing apoptosis show characteristic morphological and biochemical features, which can be readily measured and quantified. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed "secondary necrosis"

As used herein, the terms "**cell**," "**cells**," "**cell line**," "**host cell**," and "**host cells**," are used interchangeably and, encompass plant, and animal cells and include invertebrate, non-mammalian vertebrate and mammalian cells. All such designations include cell populations and progeny. Thus the terms "transformants" and "transfectants" include the primary subject cell and cell lines derived therefrom without regard for the number of transfers. Exemplary non-mammalian vertebrate cells include, for example, avian cells, reptilian cells and amphibian cells. Exemplary invertebrate cells include, but are not limited to, insect cells such as, for example, caterpillar (Spodoptera frugiperda) cells, mosquito (Aedes aegypti) cells, fruitfly (Drosophila melanogaster) cells, Schneider cells, and Bombyx mori cells See, e.g., Luckow et al., Bio/Technology 6:47-55 (1988). The cells may be differentiated, partially differentiated or undifferentiated, e.g. stem cells, including embryonic stem cells and pluripotent stem cells. Additionally tissue samples derived from organs or organ systems may be used according to the invention. Exemplary mammalian cells include, for example, cells derived from human, non-human primate, cat, dog, sheep, goat, cow, horse, pig, rabbit, rodents including mouse, hamster, rat and guinea pig and any derivatives and progenies thereof.

The terms "**cell culture**," or "**tissue culture**" refer to cells grown in suspension or grown adhered to a variety of surfaces or substrates in vessels such as roller bottles, tissue culture flasks, dishes, multi-well plates and the like. Large scale approaches, such as bioreactors, including adherent cells growing attached to microcarriers in stirred fermentors, are also encompassed by the term "cell culture." Moreover, it is possible not only to culture contact-dependent cells, but also to use suspension culture techniques in the methods of the claimed invention. Exemplary microcarriers include, for example, dextran, collagen, plastic, gelatin and cellulose and others as described in Butler, Spier & Griffiths, Animal cell Biotechnology 3:283-303 (1988). Porous carriers, such as, for example, Cytoline™ or Cytopore™, as well as dextran-based carriers, such as DEAE-dextran (Cytodex 1™ quaternary amine-coated dextran (Cytodex™) or gelatin-based carriers, such as gelatin-coated dextran (Cytodex 3™) may also be used. Cell culture procedures for both large and small-scale production of proteins are encompassed by the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, with or without microcarriers, and operated alternatively in a batch, fed-batch, or perfusion mode.

The terms "**cell culture medium**," "**cell culture media**," and "**culture medium**" refer to the solutions used for growing, storing, handling and maintaining cells and cell lines. Such solutions generally include various factors necessary for cell attachment, growth, and maintenance of the

cellular environment. For example, a typical solution may include a basal media formulation, various supplements depending on the cell type and, occasionally, antibiotics. In some embodiments, a solution may include at least one component from one or more of the following categories: 1) an energy source, usually in the form of a carbohydrate such as glucose; 2) all essential amino acids, and usually the basic set of twenty amino acids plus cystine; 3) vitamins and/or other organic compounds required at low concentrations; 4) free fatty acids; and 5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range. The solution may optionally be supplemented with one or more components from any of the following categories: 1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor; 2) salts and buffers as, for example, calcium, magnesium, phosphate, Tris, HEPES, and sodium bicarbonate; 3) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and 4) protein and tissue hydrolysates. In general, any suitable cell culture medium may be used. The medium may be comprised of serum, e.g. fetal bovine serum, calf serum or the like. Alternatively, the medium may be serum free, animal free, or protein free.

The term "**cell culture media component**" or "cell culture component" refers to any heterologous proteins used as a supplement for cell culture media.

The term "**cell culture media ingredient**" includes Cell culture media components, proteins, peptides, hormones, carbohydrates, amino acids, lipids, vitamins, antibiotics, organic and inorganic salts.

The term "**cell culture media supplement**" refers to a combination of one or multiple cell culture media components, with or without other ingredients, for addition to cell culture media.

The term "**cell culture media base**" or "**basal media**" refers to a cell culture media that may contain, for example, any or all of the following components: proteins, peptides, lipids, carbohydrates, amino acids, organic and/or inorganic salts, buffers (e.g., bicarbonate), vitamins, hormones, antibiotics, and pH indicators (e.g., phenol red). Examples of cell culture media bases that can be used in accordance with the present invention include: Dulbecco's Modified Eagle's Medium (DME), Ham's Nutrient Mixtures, MCDB Media, Minimum Essential Medium Eagle, RPMI Media, Ames' Media, BGJb Medium (Fitton-Jackson Modification), Click's Medium, CMRL-1066 Medium, Fischer's Medium, Glascow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), L-15 Medium (Leibovitz), McCoy's 5A Modified

Medium, NCTC Medium, Swim's S-77 Medium, Waymouth Medium, and William's Medium E. The cell culture media base may be supplemented using a cell culture media component or a cell culture media supplement.

The term "**cell lineage**" when referring to a stem cell culture refers to all of the stages of the development of a cell type, from the earliest precursor cell to a completely mature cell (i.e. a specialized cell).

The terms "**cell viability**" or "**viability**" refers to relative amounts of living and dead cells, present with a population of cells at any given time. Cell viability may be determined by measuring the relative numbers of living and dead cells in any given sample of the population. Cell viability may also be estimated by measuring the rate of cell proliferation of the entire population which represents the overall balance of the rates of cell growth and cell death. Rates of cell growth may also be directly measured, by counting the number of cells, and by using any number of commercially available cell proliferation assays which directly scores the rate of cell growth.

"**Conditioned medium**" refers to a cell culture medium that is obtained from a culture of a feeder cell on which embryonic stem cells can be cultured and maintained in a pluripotent state. The feeder cell depletes the conditioned medium of some components, but also enriches the medium with cell-derived material, probably including small amounts of growth factors. The term "feeder cell factor" as used herein means the cell-derived material that is released into the conditioned medium by the feeder cell. The cell factor that is released into the cell culture medium is useful in enhancing the growth of embryonic stem cells, or in the maintenance of the embryonic stem cell in a pluripotent state. The feeder cell factor can be identified and purified using techniques that are known to one skilled in the art, and are described herein.

The phrase "**conservative amino acid substitution**" or "**conservative mutation**" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag).

Examples of amino acid groups defined in this manner include: a "charged / polar group," consisting of Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic, or cyclic group," consisting of Pro, Phe, Tyr and Trp; and an "aliphatic group" consisting of Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys.

Within each group, subgroups can also be identified, for example, the group of charged / polar amino acids can be sub-divided into the sub-groups consisting of the "positively-charged sub-group," consisting of Lys, Arg and His; the negatively-charged sub-group," consisting of Glu and Asp, and the "polar sub-group" consisting of Asn and Gln. The aromatic or cyclic group can be sub-divided into the sub-groups consisting of the "nitrogen ring sub-group," consisting of Pro, His and Trp; and the "phenyl sub-group" consisting of Phe and Tyr The aliphatic group can be sub-divided into the sub-groups consisting of the "large aliphatic non-polar sub-group," consisting of Val, Leu and Ile; the "aliphatic slightly-polar sub-group," consisting of Met, Ser, Thr and Cys, and the "small-residue sub-group," consisting of Gly and Ala

Examples of conservative mutations include substitutions of amino acids within the sub-groups above, for example, Lys for Arg and vice versa such that a positive charge can be maintained; Glu for Asp and vice versa such that a negative charge can be maintained; Ser for Thr such that a free -OH can be maintained; and Gln for Asn such that a free -NH_2 can be maintained.

The term "**cytotoxicity**" refers to the cell killing property of a chemical compound (such as a chemical or protein contaminant, detergent, or toxin). In contrast to necrosis and apoptosis, the term cytotoxicity need not necessarily indicate a specific cellular death mechanism.

As used herein, the term "**decrease**" or the related terms "**decreased**," "**reduce**" or "**reduced**" refers to a statistically significant decrease. For the avoidance of doubt, the terms generally refer to at least a 10% decrease in a given parameter, and can encompass at least a 20% decrease, 30% decrease, 40% decrease, 50% decrease, 60% decrease, 70% decrease, 80% decrease, 90% decrease, 95% decrease, 97% decrease, 99% or even a 100% decrease (i.e., the measured parameter is at zero)

As used herein, the terms "**develop**", "**differentiate**" and "**mature**", as used to describe a stem cell, refer to the progression of a cell from the stage of having the potential to differentiate into at least two different cellular lineages to becoming a specialized and terminally differentiated cell Such terms can be used interchangeably for the purposes of the present application.

The term "**expression**" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, in situ hybridization to cellular RNA or by PCR. Proteins encoded by a selected sequence can be quantified by various methods including, but not limited to, e.g., ELISA, Western blotting, radioimmunoassays, immunoprecipitation, assaying for the biological activity of the protein, or by immunostaining of the protein followed by FACS analysis.

"Expression control sequences" are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, internal ribosome entry sites (IRES) and the like, that provide for the expression of a coding sequence in a host cell. Exemplary expression control sequences are described in Goeddel: Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990).

The term "**feeder cell**" refers to a culture of cells that grows in vitro and secretes at least one factor into the culture medium, and that can be used to support the growth of another cell of interest in culture. As used herein, a "**feeder cell layer**" can be used interchangeably with the term "feeder cell." A feeder cell can comprise a monolayer, where the feeder cells cover the surface of the culture dish with a complete layer before growing on top of each other, or can comprise clusters of cells.

The term "**growth phase**" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are dividing at a constant rate. During this phase, cells are cultured for a period of time, and under such conditions that cell growth is maximized. The determination of the growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. "Period of time and under such conditions that cell growth is maximized" and the like, refer to those culture conditions that, for a particular cell line, are determined to be optimal for cell growth and division. During the growth phase, cells are cultured in nutrient medium containing the necessary additives usually at about 30-40 ° C., generally about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line, for instance a mammalian cell.

The term "**heterologous** DNA" refers to DNA which has been introduced into a cell that is derived from another source, or which is from the same source but is located in a different (i.e.

non native) context. The term "**heterologous protein**" or "**recombinant protein**" refers to a protein encoded in all or part by heterologous DNA, or a protein that is expressed from expression control sequences (such as a promoter, or enhancer) created in whole or part by the heterologous DNA which activates the expression of an endogenous gene

The term "**homology**" describes a mathematically based comparison of sequence similarities which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al , (1997) Nucleic Acids Res. 25(17):3389-3402 When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used.

The term "**homologous**" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (e.g , the immunoglobulin superfamily) in the same species of animal, as well as homologous proteins from different species of animal (for example, myosin light chain polypeptide, etc.; see Reeck et al., *Cell*, 50:667, 1987) Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term "**growth factor**" refers to Amphiregulin, Angiopoietin, Betacellulin, (Bone Morphogenic protein-13, Bone Morphogenic protein-14, Bone Morphogenic protein-2, Human BMP-3, Bone Morphogenic protein-4, Human BMP-5, Bone Morphogenic protein-6, Bone Morphogenic protein-7, Human CD135 Ligand / Flt-3 Ligand, Human Granulocyte Colony Stimulating Factor (G-CSF), Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Human Macrophage Colony Stimulating Factor (M-CSF), Human Cripto-1, Human CTGF (Connective tissue growth factor), Human EGF (Epidermal Growth Factor), Human EG-VEGF (Endocrine-Gland-Derived Vascular Endothelial Growth Factor), Human Erythropoietin (EPO), Human FGF (Fibroblast Growth Factors 1-23), Human GDF-11, Human GDF-15, Human GDF-8, Human

Growth Hormone Releasing Factor (GHRF, GRF, GHRH, Growth Hormone Releasing Hormone), Human Heparin Binding Epidermal Growth Factor (HB-EGF), Human Hepatocyte Growth Factor (HGF), Human Heregulin beta 1, Human insulin, Human IGF-1 (Insulin-like Growth Factor-1), Human IGF-2 (Insulin-like Growth Factor-2), Human IGFBP-1 (Insulin-like Growth Factor Binding Protein 1), Human IGFBP-3 (Insulin-like Growth Factor Binding Protein 3), intestinal trefoil factor (ITF), Human keratinocyte growth factors 1 & 2, Human Leukemia Inhibitory Factor (LIF), Human MSP, Human Myostatin, Human Myostatin, pro (propeptide), Human NRG1, Human NGF, Human Oncostatin M, Human Osteoblast Specific Factor 1 (OSF-1, Pleiotrophin), Human PD-ECGF (Platelet-derived endothelial cell growth factor), Human PDGF, Human PlGF, Human Placental Growth Factor 1 (PLGF1), Human Placental Growth Factor 2 (PLGF2), Human SCGF-a (Stem Cell Growth Factor-alpha), Human SCGF-b (Stem Cell Growth Factor-beta), Human Stem Cell Factor (SCF) / CD117 Ligand, Human Thrombopoietin (TPO, THPO), Human Transforming Growth Factor, Human TGF-alpha (Transforming Growth Factor-alpha, TGFa), Human TGF-beta 1 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 1.2 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 2 (Transforming Growth Factor-beta2, TGFb), Human TGF-beta 3 (Transforming Growth Factor-beta3, TGFb), Human VEGF (Vascular Endothelial Growth Factor), Human VEGF-121, Human VEGF-165, and Human VEGF-A

As used herein, "**identity**" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing. Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48, 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul,

S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm can also be used to determine identity.

The terms "**immunoglobulin**" or "**antibody**" (used interchangeably herein) refers to a protein typically having a basic four-polypeptide chain structure consisting of two heavy and two light chains, the chains being stabilized, for example, by interchain disulfide bonds, which has the ability to specifically bind antigen. The term "single-chain immunoglobulin" or "single-chain antibody" (used interchangeably herein) refers to a protein having a two-polypeptide chain structure consisting of a heavy and a light chain, the chains being stabilized, for example, by interchain peptide linkers, which has the ability to specifically bind antigen. The term "domain" refers to a globular region of a heavy or light chain polypeptide comprising peptide loops (e.g., comprising 3 to 4 peptide loops) stabilized, for example, by beta-pleated sheet and/or intrachain disulfide bond. Domains are further referred to herein as "constant" or "variable", based on the relative lack of sequence variation within the domains of various class members in the case of a "constant" domain, or the significant variation within the domains of various class members in the case of a "variable" domain. Antibody or polypeptide "domains" are often referred to interchangeably in the art as antibody or polypeptide "regions". The "constant" domains of an antibody light chain are referred to interchangeably as "light chain constant regions", "light chain constant domains", "CL" regions or "CL" domains. The "constant" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "CH" regions or "CH" domains). The "variable" domains of an antibody light chain are referred to interchangeably as "light chain variable regions", "light chain variable domains", "VL" regions or "VL" domains). The "variable" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "VH" regions or "VH" domains). Immunoglobulins or antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Exemplary fragments include Fab, Fab', F(ab')2, Fabc and/or Fv fragments.

As used herein, the term "**increase**" or the related terms "**increased**", "**enhance**" or "**enhanced**" refers to a statistically significant increase. For the avoidance of doubt, the terms generally refer to at least a 10% increase in a given parameter, and can encompass at least a

20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 95% increase, 97% increase, 99% or even a 100% increase over the control value

The term "**isolated**," when used to describe the cell culture components, i.e. albumin, or transferrin related proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

The term "**maturation-specific protein promoter**" refers to a promoter exhibiting substantially up-regulated activity (greater than 25%) during seed maturation.

The term "**mature plant**" refers to a fully differentiated plant

"**Markers**" as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

Cells expressing "**markers of pancreatic endocrine lineage**" refer to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, NRx2.2, NRx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, and Pax6. Cells expressing markers characteristic of the pancreatic cell lineage include pancreatic β cells.

Cells expressing "**markers characteristic of endoderm lineage**" as used herein refer to cells expressing at least one of the following markers: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES),

DKK4, FGF17, GATA-6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

Cells expressing pluripotency markers derived by the methods of the present invention express at least one of the following pluripotency markers selected from the group consisting of: ABCG2, cripto, FoxD3, Connexin43, Connexin45, Oct4, SOX-2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tral-60, and Tral-81.

Cells expressing "**markers characteristic of mesoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, GATA-6

Cells expressing "**markers characteristics of ectoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: BMP-4, Noggin, Chordin, Otx2, Fox J3, Nestin, p63/TP73L, beta-III Tubulin

"**Monocot seed components**" refers to carbohydrate, protein, and lipid components extractable from monocot seeds, typically mature monocot seeds.

As used herein, the terms "**native**" or "**wild-type**" relative to a given cell, protein, polypeptide, nucleic acid, trait or phenotype, refers to the form in which that is typically found in nature.

The terms "**operably linked**" and "**operatively linked**," as used interchangeably herein, refer to the positioning of two or more nucleotide sequences or sequence elements in a manner which permits them to function in their intended manner. In some embodiments, a nucleic acid molecule according to the invention includes one or more DNA elements capable of opening chromatin and/or maintaining chromatin in an open state operably linked to a nucleotide sequence encoding a recombinant protein. In other embodiments, a nucleic acid molecule may additionally include one or more nucleotide sequences chosen from: (a) a nucleotide sequence capable of increasing translation; (b) a nucleotide sequence capable of increasing secretion of the recombinant protein outside a cell; and (c) a nucleotide sequence capable of increasing the mRNA stability, where such nucleotide sequences are operatively linked to a nucleotide sequence encoding a recombinant protein. Generally, but not necessarily, the nucleotide sequences that are operably linked are contiguous and, where necessary, in reading frame. However, although an operably linked DNA element capable of opening chromatin and/or

maintaining chromatin in an open state is generally located upstream of a nucleotide sequence encoding a recombinant protein; it is not necessarily contiguous with it. Operable linking of various nucleotide sequences is accomplished by recombinant methods well known in the art, e.g. using PCR methodology, by ligation at suitable restrictions sites or by annealing Synthetic oligonucleotide linkers or adaptors can be used in accord with conventional practice if suitable restriction sites are not present.

A "**plant cell**" refers to any cell derived from a plant, including undifferentiated tissue (e g., callus) as well as plant seeds, pollen, propagules, embryos, suspension cultures, meristematic regions, leaves, roots, shoots, gametophytes, sporophytes and microspores

The terms "**polynucleotide**" and "**nucleic acid molecule**," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. A nucleic acid molecule can take many different forms, e.g., a gene or gene fragment, one or more exons, one or more introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. As used herein, "DNA" or "nucleotide sequence" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides

The term "**pluripotent stem cell**" encompasses stem cells obtained from embryos, fetuses or adult tissues In one preferred embodiment, the pluripotent stem cell is an embryonic stem cell. In another embodiment the pluripotent stem cell is a fetal stem cell, such as a primordial germ

cell. In another embodiment the pluripotent stem cell is an adult stem cell

As used herein, the term "**pluripotent**" refers to a cell capable of at least developing into one of ectodermal, endodermal and mesodermal cells. As used herein the term "pluripotent" includes cells that are totipotent and multipotent. As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. The term "multipotent" refers to a cell that is not terminally differentiated.

A "**promoter**" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. As used herein, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. A transcription initiation site (conveniently defined by mapping with nuclease S1) can be found within a promoter sequence, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters can often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine- Dalgarno sequences in addition to the -10 and -35 consensus sequences.

A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources are well known in the art. Representative sources include for example, viral, mammalian, insect, plant, yeast, and bacterial cell types, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available on line or, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, the RSV promoter, the rice endosperm specific glutelin (Gt1) promoter, CaMV35S viral promoter. Inducible promoters include the Tet system, (US Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., Proc. Natl. Acad. Sci. (1996) 93 (8). 3346-3351; the T-RE$_x$™ system (Invitrogen Carlsbad, CA), LacSwitch® (Stratagene, (San Diego, CA) and the Cre-ER^T tamoxifen inducible recombinase system (Indra et al. Nuc. Acid. Res. (1999) 27 (22): 4324-4327: Nuc. Acid. Res. (2000) 28 (23): e99. US Patent No. 7 112,715: and Kramer & Fussenegger, Methods Mol. Biol (2005) 308: 123-144) or any promoter known in the art suitable for expression in the desired cells.

The term "**protein of interest**" refers to any protein which may be useful for research, diagnostic or therapeutic purposes. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. Exemplary proteins of interest include, but are not limited to, molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon, clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; members of the TNF and TNF receptor (TNFR) family, like tumor necrosis factor-alpha and -beta, CD40 ligand, Apo-2 ligand/TRAIL, DR4, DR5, DcR1, DcR2, DcR3, OPG, Fas ligand; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance, relaxin A-chain; relaxin B-chain; prorelaxin, mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase, DNase; IgE, a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TG-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins. CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin, osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; thrombopoietin (TPO); interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope, gp120; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and variants and/or fragments of any of the above-listed polypeptides; as well as antibodies against various protein antigens like CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4

receptor; cell adhesion molecules such as LFA-1, Mac1, p150 95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies), growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C; an Apo-2L receptor such as Apo-2 (DR5), DR4, DcR1, DcR2, DcR3; and variants and/or fragments of the above-identified antibodies etc. In one embodiment of the invention, a protein of interest will comprise a protein which itself is capable of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

The term "**production phase**" of the cell culture refers to the period of time during which cell growth has reached a plateau. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The term "**produced in a plant**" when used to describe the cell culture components disclosed herein means that the protein has been obtained directly or indirectly from a plant cell. With respect to recombinant proteins, the term "produced in a plant" means that the recombinant protein has been expressed within a plant cell, and then purified from host cells proteins and lipids. Proteins that have been produced in a plant may be differentiated from proteins that have purified from other organisms by at least three criteria. 1) Proteins produced in a plant will typically comprise different patterns of post-translational modifications compared to proteins produced in different organisms, which may be readily identified by mass spectrum analysis. 2) Proteins produced in a plant will typically comprise a different set of bound co-factors, lipids and contaminating proteins compared to the same protein produced in a different expression system, or purified from a different source. 3) Proteins produced in a plant may exhibit a different biological activity compared to the same protein produced or purified from a different source. The difference in activity in the protein produced in a plant may arise from a difference in the conformation, folding, aggregation state, post-translational modifications, or bound factors or co-purified contaminating proteins of the protein produced in a plant compared to a protein produced or isolated from a different organism. Differences in the specific activity of a protein produced in a plant may be readily established by routine assays using defined concentrations and assay conditions, for example, using cell growth, and factor synergism assays, as disclosed in Examples 11 to 19. Specifically a protein produced in a plant may exhibit at least at least 10 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. Preferably, a protein produced in a plant

may exhibit at least at least 20 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 30 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 40 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. Even more preferably, a protein produced in a plant may exhibit at least at least 50 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein.

The terms "**isolated**" or "**purified**" when used to describe the cell culture components disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

The term "**recombinant protein**" or "**recombinant polypeptide**" refers to an exogenous, i.e., heterologous or foreign polypeptide, to the cells producing the polypeptide.

The term "**stress**" in the context of apoptosis or cell culture refers to non-optimal conditions for tissue culture including any combination of the following; the presence of toxins, nutrient or growth factor depletion or withdrawal, hypoxia, thermal stress (temperature is too high or too low compared to the preferred range), loss of cell-cell contacts, viral infection, osmotic stress (osmolality is too high or too low compared to the preferred range), oxidative stress, cell density (cell density is too high or too low compared to the preferred range), and pH stress (pH is too high or too low compared to the preferred range).

The term "**transformation**" refers to the transfer of one or more nucleic acid molecules into a host cell or organism. Methods of introducing nucleic acid molecules into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection,

cationic lipid-mediated transfection, electroporation, scrape loading, ballistic introduction or infection with viruses or other infectious agents. "**Transformed**", "**transduced**", "**transgenic**", and "**recombinant**" refer to a host cell or organism into which a recombinant or heterologous nucleic acid molecule (e g., one or more DNA constructs or RNA, or siRNA counterparts) has been introduced. The nucleic acid molecule can be stably expressed (i.e. maintained in a functional form in the cell for longer than about three months) or non-stably maintained in a functional form in the cell for less than three months i.e. is transiently expressed. For example, "transformed," "transformant," and "transgenic" cells have been through the transformation process and contain foreign nucleic acid. The term "untransformed" refers to cells that have not been through the transformation process

The term "**transition phase**" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as pH, ion concentration, and temperature may shift from growth conditions to production conditions.

The term "**seed product**" includes, but is not limited to, seed fractions such as de-hulled whole seed, flour (seed that has been de-hulled by milling and ground into a powder) a seed extract, preferably a protein extract (where the protein fraction of the flour has been separated from the carbohydrate fraction), malt (including malt extract or malt syrup) and/or a purified protein fraction derived from the transgenic grain.

"**Seed maturation**" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

The term "**sequence similarity**" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (see Reeck et al., supra). However, in common usage and in the instant application, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 85%, and more preferably at least about 90% or at

least about 95% of the nucleotides match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm known such as BLAST, FASTA, DNA Strider, CLUSTAL, etc. An example of such a sequence is an allelic or species variant of the specific genes of the present invention. Sequences that are substantially homologous may also be identified by hybridization, e.g., in a Southern hybridization experiment under, e.g., stringent conditions as defined for that particular system.

Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acid residues are identical, or when greater than about 90% of the amino acid residues are similar (i.e., are functionally identical). Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above. The program may use the local homology algorithm of Smith and Waterman with the default values: Gap creation penalty = -(1+1/*k*), *k* being the gap extension number, Average match = 1, Average mismatch = -0.333.

As used herein and in the appended claims, the singular forms "a," "an," and "the," include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a molecule" includes one or more of such molecules, "a reagent" includes one or more of such different reagents, reference to "an antibody" includes one or more of such different antibodies, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds. (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands;. DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, In Situ Hybridization: Principles and Practice; Oxford University Press: M. J. Gait (Editor), 1984. Oligonucleotide Synthesis: A Practical Approach, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, Methods of Enzymology:

DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press; Using Antibodies: A Laboratory Manual: Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press. ISBN 0-87969-544-7); Antibodies: A Laboratory Manual by Ed Harlow (Editor) David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-3,4-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, N.Y., Marcel Dekker. ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory. ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods, compositions, reagents, cells, similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described herein. All publications mentioned herein are incorporated herein, including all figures, graphs, equations, illustrations, and drawings, to describe and disclose specific information for which the reference was cited in connection with.

I. Methods of using the supplements of the invention

Frequently, cell culture media is supplemented with serum at concentration of 5 to 10%. Serum is a complex mixture containing undefined essential matter for cell growth. Commonly used serum is fetal calf serum (FCS) and fetal bovine serum (FBS). Due to its complex nature, lack of consistency, and the potential risk for pathogen contamination, cell culture industries and regulatory agencies are seeking alternatives to adding serum to cell culture media.

Cell culture components and cell culture supplements added to basal media serve distinct functions in promoting cell growth. These protein components are typically animal-derived. With the increase in the concern of potential pathogen contamination from animal sourced material, cell culture industries are trying to develop animal-component-free media. While some successes have been made with the use of plant-based hydrolysates and recombinant proteins

produced using microbial systems, plant-based hydrolysates are an undefined mixture of components and recombinant protein from microbial sources are too expensive to use in routine cell culture. An alternative source of non-animal-derived components would be beneficial for industries that depend on cell culture techniques

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof, and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

According to further embodiments, the combination of proteins that is used in the methods of culturing cells and supplements of the invention provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, the supplements comprising one combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

In another aspect, the supplements comprises a combination of transferrin related proteins and albumin which provide a synergistic effect on cell growth and productivity.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, adding a supplement of the present

invention to the cell culture medium

The claimed supplements are useful in a wide range of applications for tissue and cell culture and recombinant protein production where they provide for significant improvements in preventing apoptosis and improving cell viability during tissue culture, and in particular in response to stress.

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. These changes include, changes to the cell membrane such as loss of membrane asymmetry and attachment, cellular blebbing cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, cytokines, which may be present to different degrees in tissue culture media. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis, and thus influence overall cell viability. A number of intracellular components, including ATP content, calcium level, and a number of apoptotic and anti-apoptotic genes also help regulate apoptosis. A cell may initiate intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. Stress inducing agents encountered during tissue culture include for example toxins, associated with tissue culture components such as endotoxins, and heavy metals that leach from plastic ware, transfection reagents (e.g. Lipofectamine and similar lipid based transfection reagents), viral transformation, nutrient and growth factor deprivation, associated with serum free culture, or cell differentiation protocols hypoxia and oxidative stress associated with high density culture in a bioreactor and increased intracellular calcium concentration, for example, by damage to the membrane caused by detergents and electroporation.

Before the actual process of cell death occurs, the apoptotic signals must overcome regulatory proteins which act as gatekeepers overseeing the activation of the apoptosis pathway. In vivo, this step allows the process to be stopped, should the cell no longer need to die. Several proteins are involved at this step, though two main mechanisms of regulation have been identified and include those associated with mitochondria functionality, and those directly involved in transducing the signal via adaptor proteins to the apoptotic mechanisms.

Cells grown under cell culture conditions may experience cellular stresses associated with

routine tissue culture procedures, as described above which may trigger apoptotic signals and increase the susceptibility of the cells to apoptosis. For example, nutrient deprivation associated with serum free culture, oxidative stress associated with high density growth in a bioreactor, the use of cytotoxic compounds associated with DNA transfection reagents, and thermal stresses associated with cryopreservation, may predispose the cell to enter apoptosis. By enhancing the ability of a cell to survive such signals it is possible to improve cell viability during these procedures, by preventing the cells commitment to cell death, thereby improving the success and utility of these approaches.

Recently a number of genes in eukaryotic cells have been identified which inhibit the onset or reduce the effects of apoptosis Some of these genes inhibit caspase dependent apoptotic pathways in the cell, and in fact transfecting cells with anti-apoptotic genes may be useful in prolonging the life and productivity of transfected cells grown under biologically demanding conditions. (US Patent Nos., 6,586,206; 7,531,327; US Patent Application US 2009/0170165; US2009/0181426).

Additionally the addition of exogenous heat shock proteins has in some cases been shown to improve the survival of cells in culture under a variety of conditions. (Novoselova et al., J. Neurochem. 94 597-606 (2005); Tidwell et al., Cell Stress & Chap 9 (1) 88-90 (2004); Guzhova et al., Cell Stress & Chap. 3 (1) 67-77 (1998); Hounenou et al., Cell Stress & Chap 1 (3) 161-166 (1996); Johnson et al., In vitro Cell. Dev Biol., 29A 807-812 (1993).

The present invention is based in part on the demonstration that plant derived recombinant cell culture component proteins surprisingly enhanced the cell growth and viability when added in specific mixtures to mammalian cells grown in culture Surprisingly some combinations of proteins provide for a synergistic effect on cell growth and productivity which provides unexpected benefits compared to the use of the individual proteins. Specifically, such supplements comprising plant derived recombinant albumin and transferrin related proteins result in improved culture viability, extended cell survival, improved rates of cell growth and improved yields of recombinant proteins produced from tissue culture bioreactors. Because the supplements show unexpectedly improved activity and stability they offer significant improvements compared to the use of standard recombinant or purified proteins.

The supplements disclosed in the present application are useful, for example, for improving cell viability and in accelerating the rate of cell growth of cells grown in culture. In one aspect, the supplements of the invention are useful for improving or enhancing the yield of the recombinant

proteins from the cell cultures. Further improvements provided by the invention are described in detail below.

In one embodiment, the present invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium

In one embodiment, the present invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media.

In one embodiment, the present invention includes a method for reducing the accumulation of lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor

In one embodiment, the present invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method of reducing time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor

In one embodiment, the present invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor

In one embodiment, the present invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium.

In one embodiment, the present invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium

In one embodiment, the present invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection.

In one embodiment, the present invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing.

In one embodiment, the present invention includes a method for improving the rate of cell growth or viability of stem cells grown in culture comprising the addition of a supplement of the present invention to the cell culture media.

In one embodiment, the present invention includes a method for improving the yield of a recombinant product produced from cells in culture comprising the addition of a supplement of the present invention to the cell culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the purification of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for reducing the proteolysis of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the bioactivity of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the stability of a

recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the assembly of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for creating a more human pattern of glycosylation of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a recombinant product produced from cells in culture with less immunogenicity, comprising the addition of a supplement comprising recombinant albumin to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In any of these methods, the supplements of the invention, by increasing host cell viability in culture (and during fermentation), provide for a simple and cost effective method to increase the yield, and or purity, bioactivity, stability and assembly of functional recombinant protein. Additionally, the supplements of the invention, by decreasing or inhibiting apoptosis in the cell culture, can decrease the number or presence of adverse proteases in the culture media and protect the expressed protein of interest against proteolytic degradation, thereby increasing the quality of the protein of interest produced, as evidenced by increased amounts of active protein, and increased yields of intact protein. Additionally Applicants have found that the supplements of the invention may protect the cells against potential adverse effects of agents like detergents, heavy metals and endotoxin contaminates present in the culture components, or protect the cells from toxic reagents introduced to the cells during transfection or cryopreservation.

In any of the claimed methods, the supplements of the invention can be added directly, or admixed, to the culture media at any convenient time, for example when changing the media, passaging the cells, or when plating out the cells at low density. Optionally, the supplement is added to the culture media at the beginning (at the time of initiating, day 0) of the cell culturing process. In one aspect the supplements of the invention may be added before an anticipated

stressful event, for example before cryopreservation, transfection or serum withdrawal, etc.

In another aspect, the supplement is added to the culture media during the culturing of the cells prior to the point when induction of typically apoptosis occurs. For example, during a large scale cell culture, induction of apoptosis can be observed on about day 3 or day 4 of the culture, and therefore, the supplement will preferably be added prior to day 3 or day 4. Optionally, a desired quantity of the supplement is added throughout, or for the duration of, the cell culture, for instance, on a daily basis for the entire fermentation. As an example, for a 5 day culture, the supplement could be added at day 0, and every 24 hours thereafter until the culture is terminated.

Accordingly in one embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a bioreactor by adding a supplement of the invention to the bioreactor. In one embodiment, the bioreactor comprises bacterial cells. In another aspect the bioreactor comprises yeast cells. In another aspect the bioreactor comprises plant cells. In another aspect the bioreactor comprises mammalian cells.

In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in bacterial cells, by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in yeast cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a plant cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in insect cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in mammalian cells by adding the supplement of the invention to the cell culture.

In another embodiment, the invention provides a method to increase the yield of the production phase of a cell culture system and thereby increase the productivity of a bioreactor by adding the supplement of the invention to the cell culture system prior to, or during the production phase of the cell culture system.

In one aspect of this method the yield of the production phase is increased by about 10%. In one aspect of this method the yield of the production phase is increased by about 20%. In one

aspect of this method the yield of the production phase is increased by about 30%. In one aspect of this method the yield of the production phase is increased by about 40%. In one aspect of this method the yield of the production phase is increased by about 50% In one aspect of this method the yield of the production phase is increased by about 60% In one aspect of this method the yield of the production phase is increased by about 70%. In one aspect of this method the yield of the production phase is increased by about 80%. In one aspect of this method the yield of the production phase is increased by about 90%. In one aspect of this method the yield of the production phase is increased by about 100% In one aspect of this method the yield of the production phase is increased by about 200% In one aspect of this method the yield of the production phase is increased by about 500%.

In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which the supplement was not added. In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which is added a supplement lacking either a transferrin related protein, or recombinant serum albumin. In one aspect, the yield is increased compared to the summed effect of the recombinant albumin and the transferrin related protein when added alone when measured under the same culture conditions.

In another embodiment, the invention provides a method to produce a protein of interest at a temperature that is elevated compared to normal growth conditions for the production of that protein, comprising the addition of a supplement of the invention to cells expressing the protein of interest.

In another embodiment, the invention provides a method to decrease the amount of aggregates formed in a cell culture expression system by aggregate prone proteins of interest comprising the addition of a supplement of the invention to the cell culture expression system, whereby the aggregation state of the protein is reduced.

In another embodiment, the invention provides a method to increase the activity of a protein of interest protein expressed by a cell by preventing the denaturation and aggregation of the recombinant protein comprising the addition of a supplement of the invention to the cell, whereby the specific activity of the protein of interest is increased.

In another embodiment, the invention provides a method to improve the expression of proteins in a cell culture expression system that are aggregation prone, cause precipitation to occur, or are toxic themselves to the cells comprising the addition of a supplement of the invention to the

cell culture expression system, whereby the expression of the protein of interest is increased. The amount of supplement to add in any of these methods will depend on various factors, for instance, the type of host cell, the cell density, protein of interest and culture conditions, etc. Determining the desired concentration of supplement to be added to the culture media is within the skill in the art and can be ascertained empirically by routine optimization and without undue experimentation.

The skilled artisan will readily appreciate that different cell types will have different magnitudes of responses to the supplement of the invention, and this will be determined, to some degree, by the amount or type of the proteins in the supplement. Additionally different densities of cells will require appropriate adjustment in the total amount of supplement as well as the concentration of the individual proteins added to the culture to account for the increased cell number. Additionally cells grown in suspension culture or via adherent culture will have different membrane surface areas available for protein entry and will typically exhibit different rates and degrees of response. Therefore, one should choose a concentration which provides for a sufficient inhibition of apoptosis, or increase in viability, or net cell growth. Typically the supplements of the invention will be added to a final concentration of about 0.1 %, about 0.5 %, about 1%, about 2%, about 3 %, about 4 %, about 5 %, about 6%, about 7%, about 8%, about 9%, about 10 %, about 11 %, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 25%, about 30%, about 40%, or about 50% wt/wt, or wt /volume.

There will typically be an upper range of concentration of the supplement beyond which further increases in cell survival do not occur. As described in the Examples below, Applicants have found that the supplements of the invention can inhibit apoptosis when added to cell cultures at a concentration of about 200 mg /L to about 2g/L, or more preferably about 200 mg/L to about 1000 mg/L, or more preferable about 250 to about 500 mg/L.

II. Supplements

The cell culture media, methods for producing supplemented cell culture media, and methods of culturing cells using fortified cell culture media in accordance with the present invention may include components derived from animal, plant, bacterial, yeast, and insect sources. According to one aspect of the invention, the components are derived from plant sources, and preferably from monocot plants. The components may be combined with inorganic salts such as NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, and $MgCl_2$ and other ingredients such as amino acids,

vitamins, growth factors, sugars, and antibiotics to form a variety of different cell culture media. According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to another aspect, one of the proteins is albumin, and at least one additional protein is selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof.

According to further embodiments, the combination of proteins that is used in the methods of producing the supplemented cell culture media provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, one combination of proteins that may be used in the methods of producing the cell culture media includes a combination of albumin with at least one other protein selected from the group consisting of lactoferrin, transferrin, melanotransferrin, and ovotransferin

In one aspect the supplements of the invention comprise plant derived recombinant human serum albumin.

The term **"albumin"**, refers to all naturally-occurring and synthetic forms of albumin. Preferably, the term "albumin" refers to recombinant albumin. In one aspect the albumin is from a vertebrate. In one aspect the albumin is from a mammal. In a further embodiment the albumin is human. In another aspect, the recombinant albumin is produced from a plant cell. In one particularly preferred embodiment the recombinant albumin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of albumin are listed below in **Table D 1.**

Table D1
Exemplary Albumin genes

Species	Gene Bank Accession number
Human	NP_000468.1
Pan troglodytes	XP_517233.2
Canis lupus familiaris	XP_855557.1
Bos taurus	NP_851335.1
Mus musculus	NP_033784.1
Rattus norvegicus	NP_599153.1
Gallus gallus	NP_990592.1

It will be understood that for the recombinant production of albumin in different species it will typically be necessary to codon optimize the nucleic acid sequence of the gene for the host organism in question. Such codon optimization can be completed by standard analysis of the preferred codon usage for the host organism in question, and the synthesis of an optimized nucleic acid via standard DNA synthesis. A number of companies provide such services on a fee for services basis and include for example, DNA2.0, (CA, USA) and Operon Technologies, (CA, USA).

The albumin may be in its native form, i.e , as different allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the albumin, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the albumin.

Fragments of native or synthetic albumin sequences may also have the desirable functional properties of the peptide from which they derived and may be used in any of the methods of the invention. The term "fragment" as used herein thus includes fragments of albumin provided that the fragment retains the biological or therapeutically beneficial activity of the whole molecule.

For example, albumin contains at least 2 high affinity multi- metal binding sites for a number of physiologically important metals ions including copper, zinc, cadmium and nickel. (Carter et al., Advances in Protein Chemistry 45 153-203 (1994); Bal et al., J. Inorg Biochem 70 (1) 33-39 (1998), Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009), US Patent No 6,787,636).

Since trace amounts of these metals are typically present in the recombinant production of albumin, a significant amount of these metal ions can be become chelated to the protein. The binding of these ions, and in particular the binding of cadmium and nickel to recombinant albumin is associated with cellular toxicity of the protein when added to cells as a tissue culture component.

Accordingly, in one aspect, the albumin of the present invention can comprise a fragment of albumin that includes the deletion of one or amino acids involved in the multi-metal binding sites of albumin. In one aspect the albumin fragment is created by the deletion of one or more amino acids at the N-terminus of the mature protein. In another aspect the albumin can comprise one or more deletions or mutations of any of the amino acids involved in the N-terminal metal binding site of albumin In one aspect, the amino acids to be deleted or mutated are independently selected from the first 10 amino acids of the mature protein.

The term "derivative" as used herein thus refers to albumin sequences or fragments thereof, which have modifications as compared to the native sequence. Such modifications may be one or more amino acid deletions, additions, insertions and/or substitutions. These may be contiguous or non-contiguous. Representative variants may include those having 1 to 20, or more preferably 1 to 15, 1 to 10, or 1 to 5 amino acid substitutions, insertions, and / or deletions as compared to any of genes listed in Tables D1. The substituted amino acid may be any amino acid, particularly one of the well-known 20 conventional amino acids (Ala (A), Cys (C), Asp (D), Glu (E); Phe (F); Gly (G); His (H); Ile (I); Lys (K); Leu (L); Met (M); Asn (N), Pro (P); Gin (Q); Arg (R); Ser (S), Thr (T); Val (V); Trp (W); and Tyr (Y)). Any such variant or derivative of albumin may be used in any of the methods of the invention.

Accordingly, the albumin of the invention can comprise amino acid deletions, insertions or mutations in any of the functional binding domains of albumin. In one aspect the albumin may comprise a mutation in a binding domain of albumin. In one aspect the mutated binding domain is a domain involved in the binding of aspirin, warfarin, diazepam, digitoxin, clofibrate, ibuprofen or AZT, as outlined is US Patent No. 5,780,593, or a multimetal binding site as outlined in Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009).

Thus, the albumin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native albumin amino acid sequences, for example, to any of the native albumin gene sequences listed in **Table D1**. Alternatively, the albumin may have an amino acid sequence having at least 30%

preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with albumin listed in **Table D1**. In a preferred embodiment, the albumin for use in any of the methods of the present invention is at least 80% identical to the mature secreted human serum albumin (**SEQ. ID. No. 1**) as shown underlined in the below (Swiss-Prot P02768):

MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF
EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP
ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF
FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV
ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
ECCEKPLLEK SHCIAEVENE EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR
RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE
QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV
LNQLCVLHEK TPVSDRVTKC CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL
SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
AASQAALGL

Fusion proteins of albumin to other proteins are also included, and these fusion proteins may enhance, activity, targeting, stability or potency.

Chemical modifications of the native albumin structure which retain or stabilize albumin activity or biological half-life may also be used with any of the methods described herein. Such chemical modification strategies include without limitation pegylation, glycosylation, and acylation (see Clark et al.: *J. Biol. Chem.* 271(36): 21969-21977, 1996; Roberts et al., *Adv. Drug. Deliv. Rev.* 54(4): 459-476, (2002); Felix et al.: *Int. J. Pept. Protein Res.* 46(3-4): 253-264, (1995); Garber Diabetes Obes. Metab. 7 (6) 666-74 (2005)) C- and N-terminal protecting groups and peptomimetic units may also be included.

Isomers of the native L-amino acids, e.g., D-amino acids may be incorporated in any of the above forms of albumin, and used in any of the methods of the invention. All such variants, derivatives, fusion proteins, or fragments of albumin are included, may be used in any of the methods claims or disclosed herein, and are subsumed under the term "albumin"

In one aspect of the supplements of the invention the albumin is recombinant human serum albumin that has been produced in a plant. In another aspect, the albumin comprises less than about 2% aggregated albumin. In another aspect the albumin comprises less than about 1 % aggregated albumin.

In one embodiment, the supplements of the invention comprise preparations of the co-purified recombinant albumin and rice hsps that are also essentially free of detergents and endotoxins which would otherwise mask or inhibit the positive impact of the hsp. In one aspect the supplements of the invention have less than about 1 EU of endotoxin, and the albumin is at least about 95 % pure. In another aspect the supplements of the invention comprise a composition comprising recombinant albumin bound to a heat shock protein. In another aspect the supplements of the invention comprise recombinant albumin and a rice hsp70 homolog. In one aspect the rice hsp70 homolog is selected from HSP70, Bip and rice stromal protein.

The terms "**heat shock protein**", "**HSP**" or "**hsp**", as used herein includes all naturally-occurring and synthetic forms of the heat shock protein super family that retain anti-apoptotic activity. Such heat shock proteins include the small heat shock proteins/HSPB family, Hsp40/DnaJ family, HSP70/ HSPA family, HSP90 / HSPC family, HSP110/ HSPH family and chapererone family, as well as peptide fragments and protein complexes of two or more heat shock proteins or nucleotide exchange factors (for example, complexes of HSP70 & HSP40) derived therefrom. The heat shock proteins may be in their native form, i.e., as different variants as they appear in nature in different species which may be viewed as functionally equivalent variants, or they may be functionally equivalent natural derivatives thereof, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical derivatives, including post-translational modifications and degradation products of the HSPs, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, oxidatized, isomerized, and deaminated variants of the HSP.

The term "**transferrin related protein**" refers to transferrin family proteins including transferrin, lactoferrin, melanotransferrin, and ovotransferin. The crystal structures of transferrin family members across all species that have been solved to date show a high degree of structural similarity, which is not surprising since transferrin, lactoferrin and ovotransferrin share 60-80% sequence identity. (Wally and Buchanan, Biometals (2007) 20 249-262). All transferrin related proteins contain two domains of around 340 residues, which are thought to have evolved from an ancient duplication event. For serum transferrin, ovotransferrin and lactoferrin each of the duplicated lobes binds one atom of Fe (III) and one carbonate anion. With a few notable exceptions each iron atom is coordinated to four conserved amino acid residues: an aspartic acid, two tyrosines, and a histidine, while anion binding is associated with an arginine and a

threonine in close proximity. (Anderson et al., (1989) J. Mol. Biol. 209 711-734).

Human transferrin and lactoferrin share over 61 % sequence identity and have similar three dimensional structures. Transferrins transport iron in the blood by picking up free ferric iron and delivering it to cells in a receptor-mediated endocytotic process in which the TF-receptor complex is internalized, iron is released in the endosome, and the complex is recycled to the cell surface where the TF is released. In contrast, no iron transport role for lactoferrin or ovotransferrin is known. By comparison, these proteins are thought to function to prevent invading bacteria from acquiring iron, by sequestering all available iron. Human transferrin releases iron at a higher pH than does human lactoferrin which likely relates to the need for human transferrin to release iron after endocytosis into the endosome, and the need for lactoferrin to hold onto iron in low pH environments such as the stomach (Baker & Backer (2004) Biometals 17 209-216)

The term **"transferrin"** refers to all naturally-occurring and synthetic forms of transferrin. In one aspect, the term "transferrin" refers to recombinant transferrin. In one aspect, the term "transferrin" refers to plasma derived transferrin. In one aspect the transferrin is from a vertebrate. In one aspect the transferrin is from a mammal. In a further embodiment the transferrin is human. In another aspect the recombinant transferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant transferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of transferrin are listed below in **Table D2**.

Table D2

Exemplary Transferrin genes

Species	Gene Bank Accession number
Homo sapiens	NP_001054.1
Canis lupus familiaris	XP_864550.1
Bos taurus	NP_803450.2
Mus musculus	NP_598738.1
Rattus norvegicus	NP_001013128.1
Gallus gallus	NP_990635.1
Danio rerio	NP_001015057.1

The transferrin may be in its native form, i.e , as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation

(e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the transferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the transferrin.

The transferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native transferrin amino acid sequences, for example, to any of the native transferrin gene sequences listed in **Table D2**. Alternatively, the transferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with transferrin listed in **Table D2**. In a preferred embodiment, the transferrin for use in any of the methods of the present invention is at least 80% identical to the mature human transferrin.

The term **"Lactoferrin"** refers to all naturally-occurring and synthetic forms of Lactoferrin. In one aspect, the term "Lactoferrin" refers to recombinant Lactoferrin. In one aspect, the term "Lactoferrin" refers to milk derived Lactoferrin. In one aspect the Lactoferrin is from a vertebrate. In one aspect the Lactoferrin is from a mammal. In a further embodiment the Lactoferrin is human. In another aspect the recombinant Lactoferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant Lactoferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of Lactoferrin are listed below in **Table D3**.

Table D3

Exemplary Lactoferrin genes

Species	Gene Bank Accession number
Homo sapiens	AAA59511.1
Sus scrofa	AAA31059.1
Camelus dromedarius	CAB53387.1
Bos taurus	AAA30610.1
Equus caballus	CAA09407.1

The Lactoferrin may be in its native form, i.e. as different apo forms or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation

(e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of Lactoferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of Lactoferrin. Lactoferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native Lactoferrin amino acid sequences, for example, to any of the native Lactoferrin gene sequences listed in **Table D3**. Alternatively, the Lactoferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with Lactoferrin listed in **Table D3**. In a preferred embodiment, the Lactoferrin for use in any of the methods of the present invention is at least 80% identical to the mature human Lactoferrin

The term "**melanotransferrin**" refers to all naturally-occurring and synthetic forms of melanotransferrin. In one aspect, the term "melanotransferrin" refers to recombinant melanotransferrin. In one aspect, the term "melanotransferrin" refers to melanotransferrin purified from a cell or tissue. In one aspect the melanotransferrin is from a vertebrate. In one aspect the melanotransferrin is from a mammal. In a further embodiment the melanotransferrin is human. In another aspect the recombinant melanotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant melanotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of melanotransferrin are listed below in **Table D4**.

Table D4

Exemplary **melanotransferrin** genes

Species	Gene Bank Accession number
Homo sapiens	AAA59992.1
Oryctolagus cuniculus	NP_001075461.1
Mus musculus	NP_038928.1
Canis lupus familiaris	XP_545158.2
Gallus gallus	CAA63003.1

The term "ovotransferrin" refers to all naturally-occurring and synthetic forms of ovotransferrin. In one aspect, the term "ovotransferrin" refers to recombinant ovotransferrin. In one aspect the ovotransferin is from a vertebrate. In one aspect the ovotransferin is from an avian. In another

aspect the recombinant ovotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant ovotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of ovotransferrin include CAA26040 1.

The term **"insulin related growth factor" or "IGF-1"** refers to all naturally-occurring and synthetic forms of insulin related growth factor, including IGF-1, IGF-1A, IGF-1B, IGF-2A, and IGF-2B. In one aspect, the term "insulin related growth factor" refers to recombinant insulin related growth factor In one aspect, the term "insulin related growth factor" refers to insulin related growth factor purified from a cell or tissue. In one aspect the insulin related growth factor is from a vertebrate. In one aspect the insulin related growth factor is from a mammal. In a further embodiment the insulin related growth factor is human. In another aspect the recombinant insulin related growth factor is produced from a plant cell. In one particularly preferred embodiment the recombinant insulin related growth factor is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of insulin related growth factor are listed below in **Table D5.**

Table D5
Exemplary insulin related growth factor genes

Species	Gene Bank Accession number
Homo sapiens	P01343 P05019 CAA01954.1 CAA40093.1 CAA40092.1
Bos taurus	NP_001071296
Mus musculus	NP_001104746.1 NP_034642.2 NP_908941.1 NP_001104745.1 NP_001104744 1
Xenopus laevis (African clawed frog)	Q90WW4
Danio rerio (zebrafish	NP_571900

The **insulin related growth factor** may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of **insulin related growth factor**, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of **insulin related growth factor** **insulin related growth factor** which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native **insulin related growth factor** amino acid sequences, for example, to any of the native **insulin related growth factor** gene sequences listed in **Table D5**. Alternatively, the **insulin related growth factor** may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with **insulin related growth factor** listed in **Table D5** In a preferred embodiment, the **insulin related growth factor** for use in any of the methods of the present invention is at least 80% identical to the mature human **insulin related growth factor**.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 5, about 1 to 10, about 1 to 15, about 1 to 20 or about 1 to 25. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 0.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 1 about 1 to 2, about 1 to 3, about 1 to 4 or about 1 to 5. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.5 to 0.8 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.5 about 1 to 0.75, about 1 to 1,0 about 1 to 1 25, about 1 to 1.5, about 1 to 1.75 or about 1 to 2.0 In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.8 to 1.5 g/L In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components,

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.25 about 1 to 0.33, about 1 to 0.66 about 1 to 1.00, about 1 to 1.33, about 1 to 1.66 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 1.0 to about 1 5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components,

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.8, about 1 to 1 or about 1 to 1 2. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0 1 to about 1.5 g/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 20, about 1 to 100, or about 1 to 200. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 500, about 1 to 1000, or about 1 to 2000. In one aspect of any of these supplements, the concentration of transferrin in the culture is

about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 3000, about 1 to 4000, or about 1 to 5000. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and albumin mixed in a ratio of transferrin to albumin of about 1 to 80, about 1 to 100 or about 1 to 120. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 3 to about 8 u g/L. In one aspect the supplement exhibits at least an 80% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of IGF-1 and recombinant albumin mixed in a ratio of IGF-1 to albumin of about 1 to 100000, about 1 to 10000, or about 1 to 1000. In one aspect of any of these supplements, the concentration of IGF-1 in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In another aspect of the invention, the supplements contain one or more additional factors selected from the group consisting of a growth factor, sodium selenate and ethanolamine

In another aspect of the invention, the growth factors are independently selected from insulin, Epidermal Growth Factor (EGF), Fibroblast Growth Factors 1-23 (FGF), Insulin-like Growth Factor-1(IGF), keratinocyte growth factors 1 & 2(KGF), and Leukemia Inhibitory Factor (LIF). In one aspect the growth factor is insulin. In another aspect the growth factor is transferrin.

In one aspect, the supplements of the invention may be prepared by mixing the isolated cell culture components in aqueous solution.

Liquids of known concentration can also be combined containing one component part A (albumin or another cell culture component), to a liquid containing part B (such as a transferrin related protein or IGF-1) to obtain a ratio that contains the desired ratios of the components Powdered, lyophilized, or otherwise dried powder (albumin) can be added directly to an aqueous solution containing the cell culture component (such as transferrin related protein) in order to obtain a ratio based on dry weight of albumin at any desired range with respect to the other cell culture components Powdered, lyophilized, or otherwise dried albumin can also be blended with the cell culture component powder on a mass to mass basis to obtain a ratio that is completely based on gravimetrics. The resulting powder can be dissolved at concentrations ranging from very low (picomolar) to very high concentrations (millimolar) in suitable buffers that are common to the art to reconstitute the cell culture components

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin and one or more transferrin related proteins and /or IGF-1. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of transferrin related protein in the composition may vary from 50% to about 0.0001% of weight of the cell culture component. In other aspects the amount of transferrin related protein in the composition may vary from about 0.01% to about 0.02%, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08%. In another aspect the amount of transferrin related protein in the composition is greater than about 0.01%, or more preferably greater than about 0.05%, or more preferably greater than about 0.1% wt /wt, or more preferably greater than about 0.2% wt/wt transferrin related protein with respect to albumin.

In one aspect of any of the claimed supplements, the recombinant albumin is essentially free of endotoxin and detergents. In another aspect the albumin has less than about 1 EU /mg of endotoxin. In yet another aspect, the albumin contains less than about 10 ppm detergent. In another aspect of any of the claimed supplements, the albumin has a purity of greater than 95 %

In another of any of the claimed supplements, the supplement comprises recombinant albumin which is bound to a rice heat shock protein, wherein the complex has less than about 1 EU of

endotoxin and is at least 95 % pure with respect to the albumin In one aspect the recombinant albumin is produced in rice.

In another aspect of any of these methods the supplement contains recombinant albumin as the cell culture component, and the albumin is essentially free of aggregated albumin. In another aspect of any of these supplements the recombinant albumin has less than about 2 % aggregated albumin

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin with one or more bound hsps. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of hsp in the albumin composition may vary from 5 % to about 0.001% of weight of albumin. In other aspects the amount of hsp in the albumin composition may vary from about 0.01% to about 0.02 %, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08 %. In another aspect the amount of hsp in the albumin composition is greater than about 0.02%, or more preferably greater than about 0.03%. or more preferably greater than about 0.04% wt /wt, or more preferably greater than about 0.05% wt/wt hsp with respect to albumin.

III. Production of Cell culture media components

Albumin and transferrin related proteins, as well as other cell culture media components for use in the supplements of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (see below), or by chemical synthesis. using, for instance, automated peptide synthesizers, or any combination of such methods The means for preparing such polypeptides are well understood in the art.

For recombinant production, host cells can be genetically engineered to incorporate nucleic acids encoding the cell culture media component. Typically the nucleic acid will be codon optimized for high level expression in the expression system of choice, and incorporated into an expression vector to enable the expression of the protein of interest in the host cell. Vectors can exist as circular, double stranded DNA, and range in size form a few kilobases (kb) to hundreds of kb. Preferred cloning vectors have been modified from naturally occurring plasmids to facilitate the cloning and recombinant manipulation of polynucleotide sequences. Many such vectors are well known in the art and commercially available; see for example, by Sambrook (In. "Molecular Cloning: A Laboratory Manual," second edition, edited by Sambrook, Fritsch, &

Maniatis, Cold Spring Harbor Laboratory, (1989)), Maniatis, In: Cell Biology: A Comprehensive Treatise. Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608(1980).

General and specific techniques for producing proteins from plant cells may be obtained from the following patents and applications, each of which is incorporated herein in its entirety by reference: U.S. Pat Pub. No. 2003/0172403 A1 ("Plant Transcription Factors and Enhanced Gene Expression"); U.S. Pat. No. 6,991,824 ("Expression of Human Milk Proteins in Transgenic Plants"); U.S. Pat Pub. No. 2003/0221223 ("Human Blood Proteins Expressed in Monocot Seeds"); U.S. Pat. Pub. No. 2004-0078851 ("Production of Human Growth Factors in Monocot Seeds"); U.S Pat. Pub. No. 2004/0063617 ("Method of Making an Anti-infective Composition for Treating Oral Infections"); and international application no. PCT/US2004/041083 ("High- level Expression of Fusion Polypeptides in Plant Seeds Utilizing Seed- Storage Proteins as Fusion Carriers"). Other general and specific techniques for producing proteins from plant cells may be obtained, for example, from the following references, each of which is incorporated herein in its entirety by reference: U.S. Patent No. 5,693,507, U.S. Patent No. 5,932,479, U.S Patent No. 6,642,053, and 6,680,426 (each titled "Genetic Engineering of Plant Chloroplasts"); U.S. Pat. Pub No. 2005/0066384 ("Site-Targeted Transformation Using Amplification Vectors"); U.S Pat Pub. No. 2005/0221323 ("Amplification Vectors Based on Trans-Splicing"); U.S. Pat. Pub. No. 2006/0026718 ("Method of Controlling Cellular Processes in Plants"); and U.S. Pat. Pub. No. 2006/0075524 (Method of Controlling A Cellular Process in a Multi- Cellular Organism"), Marillonnet et at., Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants, Nature Biotech. (2005) 23(6): 718-723.

Expression vectors include plasmids, episomes, cosmids retroviruses or phages; the expression vector can be used to express a DNA sequence encoding the cell culture media component, and in one aspect comprises an assembly of expression control sequences. The choice of promoter and other regulatory elements can vary according to the intended host cell, and many such elements are available commercially, and can be readily assembled from isolated components such as the Gateway system from Invitrogen, (CA, USA). Expression systems for cell culture media components can be stable or transient expression systems.

Representative commercially available viral expression vectors include, but are not limited to, the adenovirus-based systems, such as the Per.C6 system available from Crucell, Inc., lentiviral-based systems such as pLP1 from Invitrogen, and retroviral vectors such as tobacco mosaic virus based vectors (Lindbo et al., BMC Biotechnol (2007) 7 52-56)

An episomal expression vector is able to replicate in the host cell, and persists as an extrachromosomal episome within the host cell in the presence of appropriate selective pressure. (See for example, Conese et al., Gene Therapy 11: 1735-1742 (2004)). Representative commercially available episomal expression vectors include, but are not limited to, episomal plasmids that utilize Epstein Barr Nuclear Antigen 1 (EBNA1) and the Epstein Barr Virus (EBV) origin of replication (oriP), specific examples include the vectors pREP4, pCEP4, pREP7 from Invitrogen. The host range of EBV based vectors can be increased to virtually any eukaryotic cell type through the co-expression of EBNA1 binding protein 2 (EPB2) (Kapoor et al., EMBO. J. 20: 222-230 (2001)). vectors pcDNA3.1 from Invitrogen, and pBK-CMV from Stratagene represent non-limiting examples of an episomal vector that uses T-antigen and the SV40 origin of replication in lieu of EBNA1 and oriP.

An integrating expression vector can randomly integrate into the host cell's DNA, or can include a recombination site to enable the specific recombination between the expression vector and the host cells chromosome. Such integrating expression vectors can utilize the endogenous expression control sequences of the host cell's chromosomes to effect expression of the desired protein. Examples of vectors that integrate in a site specific manner include, for example, components of the flp-in system from Invitrogen (e.g., pcDNA™5/FRT), or the cre-lox system, such as can be found in the pExchange-6 Core Vectors from Stratagene. Examples of vectors that integrate into host cell chromosomes in a random fashion include, for example, pcDNA3.1 (when introduced in the absence of T-antigen) from Invitrogen, pCI or pFN10A (ACT) Flexi® from Promega.

Alternatively, the expression vector can be used to introduce and integrate a strong promoter or enhancer sequences into a locus in the cell so as to modulate the expression of an endogenous gene of interest (Capecchi MR. Nat Rev Genet. (2005), 6 (6):507-12. Schindehutte et al., Stem Cells (2005); 23 (1):10-5). This approach can also be used to insert an inducible promoter, such as the Tet-On promoter (US Patents 5,464,758 and 5,814,618), in to the genomic DNA of the cell so as to provide inducible expression of an endogenous gene of interest. The activating construct can also include targeting sequence(s) to enable homologous or non-homologous recombination of the activating sequence into a desired locus specific for the gene of interest (see for example, Garcia-Otin & Guillou, Front Biosci. (2006) 11:1108-36). Alternatively, an inducible recombinase system, such as the Cre-ER system, can be used to activate a transgene in the presence of 4-hydroxytamoxifen (Indra et al. Nuc Acid. Res. (1999) 27 (22): 4324-4327; Nuc Acid. Res. (2000) 28(23): e99; and U.S. Patent No 7,112,715).

Suitable cells for producing the cell culture media components include prokaryotic cells, yeasts, insect cells, plant expression systems and mammalian expression systems. Within these general guidelines, useful microbial hosts include, but are not limited to, bacteria from the genera Bacillus, Escherichia (such as *E. coli*), Pseudomonas, Streptomyces, Salmonella, Erwinia, *Bacillus subtilis*, *Bacillus brevis*, the various strains of *Escherichia coli* (e.g., HB101, (ATCC NO. 33694) DH5α, DH10 and MC1061 (ATCC NO. 53338)).

Many strains of yeast are known to those skilled in the art and are also available as host cells for the expression of cell culture media components including those from the genera Hansenula, Kluyveromyces, Pichia, Rhino-sporidium, Saccharomyces, and Schizosaccharomyces, and other fungi. Preferred yeast cells include, for example, *Saccharomyces cerivisae* and *Pichia pastoris*.

Additionally, where desired, insect cell systems can be utilized to produce the cell culture media components for use in the supplements and methods of the present invention. Such systems are described, for example, by Kitts et al., Biotechniques, 14:810-817 (1993); Lucklow, Curr. Opin. Biotechnol., 4:564-572 (1993); and Lucklow et al. (J Virol., 67:4566-4579 (1993). Preferred insect cells include Sf-9 and Hi5 (Invitrogen, Carlsbad, Calif.).

Many suitable plant expression systems can be used for the expression of cell culture media components examples includes for example, any monocot or dicot plant. Suitable monocot plants include without limitation, rice, barley, wheat, rye, corn, millet, triticale, or sorghum, preferably rice. Other suitable plants include Arabidopsis, Alfalfa, tobacco, peanut and soybean.

In general, expression vectors for use in plants include the following operably linked elements that constitute a chimeric gene: a promoter derived from a gene encoding a plant protein, operatively linked to a gene encoding a cell culture media component. The promoter region is chosen to be regulated in a manner allowing for induction under seed-maturation conditions. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, corn, millet, triticale or sorghum.

In one aspect, the chimeric gene includes a promoter which exhibits increased expression during seed maturation. Examples of such promoters include the maturation-specific promoter region associated with one of the following maturation-specific monocot plant storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

In one aspect, the chimeric gene includes i) a promoter from the gene of a maturation-specific monocot plant storage protein, ii) a first DNA sequence, operably linked to the promoter, encoding a monocot plant seed-specific signal sequence (such as an N-terminal leader sequence or a C-terminal trailer sequence) capable of targeting a polypeptide linked thereto to an endosperm cell, preferably an endosperm-cell organelle, such as a protein storage body, and iii) a second DNA sequence, linked in translation frame with the first DNA sequence, encoding a cell culture media component. The signal sequence is preferably cleaved from the cell culture media component in the plant cell.

The chimeric gene, in turn, is typically placed in a suitable plant-transformation vector having (i) companion sequences upstream and/or downstream of the chimeric gene which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the desired plant host; (ii) a selectable marker sequence; and (iii) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells.

At least one selective purification tag and/or at least one specific protease cleavage site may be provided for eventual release of the cell culture media component from the monocot seed storage protein carrier. For example, a strategic methionine or tryptophan residue providing a chemical cleavage site may be engineered in frame between the domains for release of the cell culture media component from the endogenous plant protein. Other selective protease cleavage sites include, but are not limited to enterokinase (ek), Factor Xa, thrombin, V8 protease, GENENASE™(a variant of subtilisin BPN'), α-lytic protease or tobacco etch virus protease. Alternatively, cleavage of the fusion protein could be performed via chemical cleaving agents such as cyanogen bromide or N-chlorosuccinimide.

A number of suitable mammalian host cells are also known in the art and many are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209 Examples include, but are not limited to, mammalian cells, such as Chinese hamster ovary cells (CHO) (ATCC No. CCL61) CHO DHFR-cells (Urlaub et al., Proc. Natl. Acad Sci. USA, 97·4216-4220 (1980)), human embryonic kidney (HEK) 293 or 293T cells (ATCC No CRL1573), or 3T3 cells (ATCC No. CCL92). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art Other suitable mammalian cell lines are the

monkey COS-1 (ATCC No. CRL1650) and COS-7 cell lines (ATCC No. CRL1651), and the CV-1 cell line (ATCC No. CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines.

Cell-free transcription and translation systems can also be employed to produce such proteins using the DNA constructs (or RNAs derived from the DNA constructs) of the present invention.

Production of recombinant proteins of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides encoding the cell culture media component and to host cells which are genetically engineered with such expression systems and to the production of such proteins by recombinant techniques. In one embodiment the host cell endogenously expresses a heat shock protein of interest.

In cases where purification of the expressed proteins of the supplement of the invention are necessary, proteins of the present invention can be recovered from either the cellular environment, before lysing the cells, or after cell lysis. The proteins can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. High performance liquid chromatography is also employed for purification.

A search of patents, published patent applications, and related publications will provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying albumin. For example, US Patent Nos. 4,075,197; 4,086,222; 4,093,612; 4,097,473; 4,136,094; 4,228,154; 5,250,662; 5,656,729; 5,677,424; 5,710,253; 5,728,553; 5,994,507; 6,001,974; 6,638,740; 6,617,133 and 7,423,124 disclose various processes for purifying albumin. In one aspect, the albumin for use in the present invention is purified using any of these art recognized processes listed above, and then mixed in aqueous solution with a heat shock protein. Methods for the purification of heat shock proteins, including anion exchange chromatography and ATP agarose affinity chromatography are well known in the art. (Welch & Feramisco, J Biol. Chem 257 (24)14949-14959; (1982); Welch & Feramisco, Mol. Cell Biol 5 (6) 1229-1237 (1985). Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis,

isolation and/or purification

In one preferred aspect recombinant albumin is purified using procedures that enable the direct co-purification of both recombinant albumin and a heat shock protein, or hsp protein complex, for example as described below and in the Examples section. In one aspect the recombinant albumin is produced in rice, and the heat shock protein is an endogenous rice heat shock protein

In one aspect, of this method, expression vectors are used to increase the expression of the recombinant albumin in the host cell, while the expression of the host cells endogenous heat shock proteins is accomplished by activating the expression of the host cells hsp genes. In another aspect expression vectors are used to increase the expression of the heat shock protein In another aspect expression vectors are used to increase the expression of the heat shock protein and albumin. In another aspect the nucleic acid sequence encoding a heat shock protein and albumin are located in the same expression vector.

Due to the similar electronegativity of albumin and hsp70, anion exchange chromatography is the preferred method to prepare albumin enriched in Hsps. For example, both albumin and Hsp70 bind to anion exchange columns with resins consisting of either quaternary amine or diethylaminoethyl mounted on a bead that is suitable for the ion exchange of polypeptides (large molecular exclusion limit and of suitable size) at high pH (7.5 and above). Examples of such resins are General Electric (GE) Q Sepharose and GE DEAE Sepharose. Due to their similar electronegativity, utilizing low pH conditions (below pH 6.5) allows for the co-purification of the two molecules on cation exchangers as well. Examples of such cation exchangers are GE Carboxymethyl Sepharose and Sulfonic acid Sepharose based resins Because the albumin and Hsp70 have similar isoelectric points, mixed mode resins may also be employed for the co-purification of albumin and Hsp70. Since both Hsp70 and Albumin are well known to bind to fatty acids and other hydrophobic molecules, it is also possible to co-purify albumin and Hsp70 on a hydrophobic based resin such as octyl sepharose (GE). Due the similar size of Hsp70 proteins and Albumin (65-75 kDa), co-purification of the two proteins and enrichment of Hsp70 by tangential flow ultrafiltration utilizing both higher and lower molecular exclusions than 65-75 kDa may also be employed to co-purify and thus enrich Albumin with hsps.

Also due to their similar molecular weights, any method that separates polypeptides based on size should effectively co-purify albumin and hsp70 such as molecular sieves and gel filtration or size exclusion chromatography. In addition, due to the similar nature of Hsp70 and Albumin in

terms of hydrophobicity and electronegativity or surface charge may be co-purified by precipitation under a number of conditions. Some of those conditions are precipitation by ammonium sulfate, precipitation by denaturants such as urea, or precipitation based on isoelectric point and solubility.

The methods are also applicable to enrich albumin with hsps from other sources. For example albumin derived from native and transgenic animal feedstock serum, as well as albumin produced from recombinant organisms and tissue culture systems based on prokaryotic and eukaryotic cells, including, vertebrate cells such as mammalian cells, and non vertebrate cells, such as insects, as well as plant, and fungi such as yeast, and the like.

A search of patents, published patent applications, and related publications will also provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying transferrin related proteins. For example, US Patent Nos 7,368,141; 7,354,902; 5,708,149; 5,596,082; 5,169,936; 4,791,193; and 4,667.018 disclose various processes for purifying transferrin related proteins

IV Exemplary Cells

Without wishing to be bound by theory, it is contemplated that any cell which is susceptible to apoptosis may be used in the methods of the invention, including primary cells, immortalized cells, differentiated cells, undifferentiated cells or cells, such as stem cells, with varying degrees of specialization. In a particular embodiment, cells used in the methods of the invention are transfected with a nucleic acid molecule comprising a nucleotide sequence encoding a protein of interest, e.g., a therapeutic protein or an antibody

In a particular embodiment, the cells used in the methods of the invention are eukaryotic cells, e.g., mammalian cells. Examples of mammalian cells include, but are not limited to, for example, human B-cells, and T cells, and derivatives thereof, such as hybridomas, and cell expressing markers of B or T cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc Natl. Acad. Sci. USA, 77:4216 (1980)); CHO-K1 cell (ATCC CCL-61), human PER.C6 cells (Crucell, NV), mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-

1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; FS4 cells; NSO mouse myeloma cells (ECACC; SIGMA), and a human hepatoma line (Hep G2). Additional examples of useful cell lines include, but are not limited to, HT1080 cells (ATCC CCL 121), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB carcinoma cells (ATCC CCL 17), 2780AD ovarian carcinoma cells (see Van der Blick, A. M. et al., Cancer Res. 48:5927-5932 (1988), Raji cells (ATCC CCL 86), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells (ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607), Wi-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. These and other cells and cell lines are available commercially, for example from the American Type Culture Collection (Virginia, USA). Many other cell lines are known in the art and will be familiar to the ordinarily skilled artisan; such cell lines therefore can be used equally well in the methods of the present invention. In a particular embodiment, cells used in the methods of the invention are CHO cells or NSO cells. Hybridomas and antibody-producing cells may also be used in the methods of the invention

In another embodiment, cells used in any of the methods of the invention are stem cells. Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate in vitro into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts

Types of human embryonic stem cells that may be used in any of the methods of the invention include established lines of human embryonic cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the

initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga.). In one embodiment, Human embryonic stem cells are prepared as described by Thomson et al. (U.S. Pat. No. 5,843,780; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff., 1998; Proc. Natl Acad. Sci U.S.A. 92:7844, 1995).

Additionally, hybridoma cells can also be used in the methods of the invention. The term "hybridoma" refers to a hybrid cell line produced by the fusion of an immortal cell line of immunologic origin and an antibody producing cell. The term encompasses progeny of heterohybrid myeloma fusions, which are the result of a fusion with human cells and a murine myeloma cell line subsequently fused with a plasma cell, commonly known as a trioma cell line. Furthermore, the term is meant to include any immortalized hybrid cell line which produces antibodies such as, for example, quadromas. See. e.g., Milstein et al., Nature, 537:3053 (1983). The hybrid cell lines can be of any species, including human, rabbit and mouse.

In some embodiments, a cell line used in the methods of the invention is an antibody-producing cell line. Antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan See, e.g., Current Protocols in Immunology, Coligan et al., Eds., Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements. In general, any cell suitable for recombinant protein expression in cell culture can be used in the methods of the invention.

In some embodiments, the cells used in the methods of the present invention may include a heterologous nucleic acid molecule which encodes a desired recombinant protein, e.g., a therapeutic protein or antibody which is desired to be produced using the methods of the invention. In a particular embodiment, the methods of the present invention are useful for producing high titers of a desired recombinant protein, e.g., a therapeutic protein or antibody, in the presence of reduced levels of one or more contaminants.

V. Cell Culture Media

Any suitable culture medium or feed medium suitable for cell growth and protein production may be used in the methods of the invention Suitable culture or feed mediums are chosen for their

compatibility with the host cells and process of interest Suitable culture or feed mediums are well known in the art and include, but are not limited to, commercial media such as Ham's F10 (SIGMA), Minimal Essential Medium (SIGMA), RPMI-1640 (SIGMA), and Dulbecco's Modified Eagle's Medium SIGMA) are suitable for culturing the animal cells In addition, any of the media described in Ham and Wallace, (1979) Meth. Enz., 58:44; Barnes and Sato,(1980) Anal. Biochem., 102:255; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469 or 4,560,655; International Publication Nos. WO 90/03430; and WO 87/00195 may be used.

Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The necessary growth factors for a particular cell are readily determined empirically without undue experimentation, as described for example in Mammalian Cell Culture (Mather, J. P ed., Plenum Press, N.Y (1984), and Barnes and Sato, Cell, 22:649 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the protein of interest in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed (IRL Press, 1991).

VI. Exemplary Cell Culture Expression Products

In one aspect of any of the claimed methods, the supplements of the invention are used to improve the viability and growth of a cell which is used to express and produce a protein of interest. The cell may express the protein of interest endogenously or may be an engineered cell line that has been modified genetically to express the protein of interest at levels above background for that cell.

Cells may be genetically modified to express a protein by transformation with a nucleic acid encoding the protein of interest, or by transformation of an activating sequence that promotes the expression of an endogenous gene In one aspect the protein of interest may be expressed from an expression vector, in which a coding sequence for the protein of interest is operably linked to an expression control sequences, to enable either constitutive or inducible expression, as is known in the art.

The protein of interest may be any protein, or fragment thereof, which is of commercial, therapeutic or diagnostic value including without limitation cytokines, chemokines, hormones, antibodies, anti-oxidant molecules, engineered immunoglobulin-like molecules, a single chain antibodies, a humanized antibodies, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, transdominant negative mutants of a target protein, toxins, conditional toxins, antigens, a tumor suppresser proteins, growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). The protein of interest may also comprise pro-drug activating enzymes

In some embodiments, the protein of interest comprises a glycoprotein, or any other protein which has one or more post-translational modifications. For example, any protein which is suitable for production in a eukaryotic host may be expressed using the methods and compositions described here.

The methods of the invention can be used to produce any desired recombinant protein or fragment thereof. In some embodiments, a recombinant protein produced using the methods described herein is a therapeutic protein. In other embodiments, the recombinant protein is an antibody or functional fragment thereof. Antibodies which may be produced using the methods of the invention include, for example, polyclonal, monoclonal, monospecific, polyspecific, fully human, humanized, single-chain, chimeric, hybrid, CDR grafted,. It may comprise a full length IgG1 antibody or an antigen-binding fragments thereof, such as, for example, Fab, $F(ab')_2$, Fv, and scfv.

Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN™) (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992), U.S Pat No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN™), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108, B1, or Tositumomab (BEXXAR™.); anti-IL-8 (St

John et al., Chest, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN™ (Kim et al., Growth Factors, 7:53-64 (1992). International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15. 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., Transplant Intl. 4:3-7 (1991), and Hourmant et al., Transplantation 58.377-380 (1994)); anti-IgE (Presta et al., J. Immunol. 151.2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-alpha, antibodies including cA2 (REMICADE™), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. J. Immunol. 156(4):1646-1653 (1996), and Dhainaut et al. Crit. Care Med. 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human alpha 4 beta 7 integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985), anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT™) and (ZENAPAX™) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997), anti-CD4 antibodies such as the cM-7412 antibody (Choy et al. Arthritis Rheum 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. Nature 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fc gamma RI as in Graziano et al. J. Immunol. 155(10).4996-5002 (1995), anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. Cancer Res. 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. Cancer Res. 55(23). 5852s-5856s (1995); and Richman et al. Cancer Res. 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. Eur J. Immunol. 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. J. Immunol. 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. Cancer Res 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. Cancer Res 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX™.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO™ anti-RSV antibodies such as MEDI-493 (SYNAGIS™.); anti-CMV antibodies such as PROTOVIR™.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as

the anti-Hep B antibody OSTAVIR™; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-.alpha.v.beta.3 antibody VITAXIN™; anti-human renal cell carcinoma antibody such as ch-G250, ING-1; anti-human 17-1A antibody (3622W94), anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40

The recombinant protein may be a cellular protein such as a receptor (e.g., membrane bound or cytosolic) or a structural protein (e.g. a cytoskeleton protein) The recombinant protein may be cellular factor secreted by the cell or used internally in one or more signal transduction pathways. Non limiting examples include, but are not limited to, CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1, IL-2, IL-3, IL-7, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF, EGF receptor, VEGF receptor, hepatocyte growth factor, osteoprotegerin ligand, interferon gamma, B lymphocyte stimulator C5 complement TAG-72, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2, and CTLA4 (which is a cytotoxic T lymphocyte-associated antigen).

The recombinant protein may also be derived from an infectious agent such as a virus, a bacteria, or fungus. The protein may be derived from a bacterial membrane or cell wall, or may be derived from the bacterial cytosol. The protein may be a bacterial or yeast enzyme, transcription factor, or structural protein. The bacterial or yeast protein may be membrane bound, cytsolic, or secreted. Examples of infectious agents include, but are not limited to, Streptococcus mutans, and Staphylococcus aureus, and Candida albicans.

The methods of the invention can also be used to produce recombinant fusion proteins comprising all or part of any of the above-mentioned proteins. For example, recombinant fusion proteins comprising one of the above-mentioned proteins plus a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an antibody, or a substantially similar protein, can be produced using the methods of the invention. See e.g. International Application No. WO 94/10308; Lovejoy et al. (1993), Science 259:1288-1293; Harbury et al. (1993), Science 262:

1401-05: Harbury et al (1994), Nature 371:80-83; Hang.kansson et al (1999), Structure 7:255-64.

Also encompassed by this invention are pharmaceutical compositions including one or more recombinant proteins produced by the methods described herein. In some embodiments, pharmaceutical compositions further include a pharmaceutically acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a subject

VII Stem Cells

In one embodiment, human embryonic stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation, comprising a supplement of the invention. The growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type and further comprising a supplement of the present invention. Alternatively, the growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a chemically defined medium comprising a supplement of the present invention. Examples of feeder-free, serum free culture systems in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal include those disclosed in US patent applications, US20050148070, US20050244962, US20050233446, U.S. Pat. No.6,800,480, and PCT publications WO2005065354 and WO2005086845

In an alternate embodiment, human embryonic stem cells are initially cultured with a layer of feeder cells that support the human embryonic stem cells and further comprising a supplement of the present invention The human embryonic are then transferred to a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation and which further comprises a supplement of the present invention. In any of these approaches, the use of the supplements of the invention results in significantly enhanced rates of cell growth and improved cell viability.

Examples of conditioned media suitable for use with the supplements of the present invention are disclosed in US20020072117, U.S. Pat. No. 6,642,048, WO2005014799, and Xu et al (Stem

Cells 22: 972-980, 2004). An example of a chemically defined medium suitable for use with the supplements of the present invention may be found in US20070010011.

Examples of feeder cells include feeder cells selected from the group consisting of a fibroblast cell, a MRC-5 cell, an embryonic kidney cell, a mesenchymal cell, an osteosarcoma cell, a keratinocyte, a chondrocyte, a Fallopian ductal epithelial cell, a liver cell, a cardiac cell, a bone marrow stromal cell, a granulosa cell, a skeletal muscle cell, a muscle cell and an aortic endothelial cell. In a preferred embodiment, the MRC-5 cell, has ATCC Catalog Number 55-X; the transformed and has ATCC Accession Number CRL-2309; the human osteosarcoma cell has ATCC Accession Number HTB-96; and the mesenchymal cell is a human fetal palatal mesenchymal cell with ATCC Accession Number CRL-1486. In other preferred embodiments the human fibroblast cell is a skin keloid fibroblast. KEL FIB and has ATCC Accession Number CRL-1762, or is a fetal skin fibroblast cell; and the bone marrow stromal cell, HS-5, has ATCC Accession Number CRL-11882.

Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium, 200 mM L-glutamine, Gibco #15039-027, non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522; human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

In one embodiment, the human embryonic stem cells are plated onto a suitable culture substrate that is treated prior to treatment according to the methods of the present invention, with a composition comprising a supplement of the present invention. In one embodiment, the treatment is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL (Becton Dickenson). MATRIGEL is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

Other extracellular matrix components and component mixtures are suitable as an alternative and can be used with the supplements of the present invention. This may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations with a supplement of the present invention.

In another embodiment, the invention encompasses an embryonic stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention. In one embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention.

In another embodiment the invention encompasses an embryonic stem cell culture, comprising a human embryonic stem cell and a human feeder cell culture comprising a supplement of the invention. In another embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a human feeder cell culture comprising a supplement of the invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing pluripotency markers, comprising the steps of:

Culturing human embryonic stem cells;

Differentiating the human embryonic stem cells into cells expressing pluripotency markers, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing markers, characteristic of ectodermal, endodermal or mesodermal cells, comprising the steps of:

Culturing pluripotency stem cells;

a. Differentiating the pluripotency stem cells into cells expressing markers characteristic of ectodermal, endodermal or mesodermal cells, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In any of these methods, the stem cells can be differentiated into cells expressing markers characteristic of an endodermal, ectodermal or mesodermal lineage by any method in the art. For example, cells expressing pluripotency markers may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al. Nature Biotechnology 23, 1534-1541 (2005), by Shinozaki et al. Development 131, 1651-1662 (2004), McLean et al., Stem Cells 25, 29-38 (2007), D'Amour et al., Nature Biotechnology 24, 1392-1401 (2006).

Cells expressing markers characteristic of the endoderm lineage may be further differentiated

into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art. For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 24, 1392-1401 (2006), wherein the differentiation is conducted in the presence of a supplement of the present invention

In one aspect of any of these methods of differentiation, the human embryonic stem cells are cultured and differentiated on a tissue culture substrate coated with an extracellular matrix. The extracellular matrix may be a solubilized basement membrane preparation extracted from mouse sarcoma cells (which is sold by BD Biosciences under the trade name MATRIGEL). Alternatively, the extracellular matrix may be growth factor-reduced MATRIGEL. Alternatively, the extracellular matrix may be fibronectin. In an alternate embodiment, the human embryonic stem cells are cultured and differentiated on tissue culture substrate coated with human serum. In one aspect, the tissue culture substrate is coated with extracellular matrix and a supplement of the present invention.

The extracellular matrix may be diluted prior to coating the tissue culture substrate. Examples of suitable methods for diluting the extracellular matrix and for coating the tissue culture substrate may be found in Kleinman, H. K., et al., Biochemistry 25:312 (1986), and Hadley, M. A., et al , J. Cell. Biol 101:1511 (1985).

In one aspect of the methods of stem cell differentiation, the culture medium should contain sufficiently low concentrations of certain factors to allow the differentiation of human embryonic stem cells to cells of endoderm, ectoderm or mesoderm lineage, such as, for example insulin and IGF (as disclosed in WO2006020919). This may be achieved by lowering the serum concentration, or alternatively, by using chemically defined media that lacks insulin and IGF. Examples of chemically defined media are disclosed in Wiles et al (Exp Cell Res. 1999 Feb. 25; 247(1): 241-8.) In a preferred embodiment, of any of these methods, the culture media comprises a supplement of the present invention.

The culture medium may also contain at least one other additional factor that may enhance the formation of cells expressing markers characteristic of endoderm, mesoderm or ectoderm lineage from human embryonic stem cells. The at least one additional factor may be, for example, nicotinamide, members of TGF-β family, including TGF-β1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB,

platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (GDF-5, -6, -8, -10, 11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS, noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine 1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA), keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof. In a preferred embodiment, of any of these methods, the culture media containing at least one additional factor listed above, further comprises a supplement of the present invention.

The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No. HTB-79), BxPC-3 (ATCC No. CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241). In a preferred embodiment, of any of these methods, the conditioned media further comprises a supplement of the present invention.

In another embodiment, the invention encompasses a method of using the cell or tissue of any of the aforementioned stem cells for the experimental, therapeutic and prophylactic treatment of a disease or condition in a human or animal. Preferably, the disease is selected from the group consisting of Parkinson's, Alzheimer's, Multiple Sclerosis, spinal cord injuries, stroke, macular degeneration, burns, liver failure, heart disease, diabetes, Duchenne's muscular dystrophy, osteogenesis imperfecta, osteoarthritis, rheumatoid arthritis, anemia, leukemia, breast cancer, solid tumors, and AIDS. In a preferred embodiment, the disease is Parkinson's or Alzheimer's. In a more preferred embodiment, the disease is Parkinson's.

VIII. Large Scale production of recombinant proteins

In one embodiment, the supplements of the present invention can be used to produce a protein of interest by growing host cells in the presence of the supplement. In one embodiment, the cell culture is performed in a stirred tank bioreactor system and a fed batch culture procedure is employed. In another embodiment a wave disposable bioreactor is employed. In the bioreactor

system, the size of the bioreactors are sufficiently large to produce the desired amount of protein of interest, such as 1,000 Liter or 12,000 Liter sizes, but are not limited to such sizes as much smaller (i.e., 2 Liter, 400 Liter) or larger (i.e., 25,000 Liter, 50,000 Liter) bioreactor vessels may be appropriate. In the preferred fed batch culture, the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process but at the termination of the culture process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cultured cells may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single step or multiple step culture procedure. In a single step culture, the host cells are inoculated into a culture environment and the method steps of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture, cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium comprising a supplement of the present invention suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase, cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan Generally, the pH is adjusted to a level between about 6.5 and 7 5 using either an acid (e.g., CO_2) or a base (e.g , Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian

cells such as CHO cells is between about 30 to 38° C. and preferably about 37° C. and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above, the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell culture environment during the production phase of the cell culture is controlled. According to the steps of the presently disclosed methods, the addition of the supplements of the invention can be coordinated such that the desired content and quality of the protein of interest is achieved and maintained in the resulting cell culture fluid. In a preferred aspect, the production phase of the cell culture is preceded by a transition phase of the cell culture in which the addition of the supplements of the invention initiates the production phase of the cell culture.

In any of the above-described methods, it is contemplated that it may be desirable to include a desired amount of agent like butyrate or Trichostatin A in the cell culture medium in combination with a supplement of the invention. Various forms of butyrate and its salts are known in the art, such as butyric acid and sodium butyrate, and are publicly available from sources such as Sigma Chemical Co. Butyrate has been reported in the literature to enhance the productivity and protein expression of cell cultures [Arts et al., Biochem J., 310:171-176 (1995), Gorman et al., Nucleic Acids Res., 11:7631-7648 (1983); Krugh, Mol. Cell. Biochem. 42:65-82 (1982); Lamotte et al., Cytotechnology, 29:55-64 (1999); Chotigeat et al., Cytotechnology, 15:217-221 (1994)]. Trichostatin A (TSA) is an inhibitor of histone deacetylase and may act similarly to butyrate in enhancing the productivity and protein expression in cell cultures [Medina et al., Cancer Research, 57:3697-3707 (1997)]. Although butyrate has some positive effects on protein expression, it is also appreciated in the art that at certain concentrations, butyrate can induce apoptosis in the cultured cells and thereby decrease viability of the culture as well as viable cell density [Hague et al., Int. J. Cancer, 55:498-505 (1993); Calabresse et al., Biochim. Biophys. Res. Comm., 195:31-38 (1993); Fillipovich et al., Biochim Biophys. Res. Comm., 198:257-265 (1994); Medina et al., Cancer Research, 57:3697-3707 (1997)]. In the methods of the present invention, a desired amount of butyrate or TSA may be added to the cell culture at the onset of the production phase and more preferably, may be added to the cell culture after a temperature shift has been implemented. Butyrate or TSA can be added in a desired amount determined empirically by those skilled in the art, but preferably, butyrate is added to the cell culture at a concentration of about 1 to about 25 mM, and more preferably, at a concentration of about 1 to about 6 mM.

Expression of the protein of interest may be measured in a sample directly, for example, by ELISA, conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorophors or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Many are commercially available.

The supplements claimed herein can also be used to increase transfection efficiency and viability of cells during transfection. Conditions and reagents used in various transfection techniques, such as Lipofectamine are relatively toxic to the cells, while electroporation can severely stress a cell. The use of higher concentrations of transfection reagents, and more extensive electroporation conditions is preferred to achieve higher transfection efficiencies. Thus the addition of the supplements of the invention prior, with, and after transfection can result in higher transfection efficiencies, and higher yields of recombinant proteins.

The supplements of the invention can be used to express proteins of interest which induce

apoptosis, such as Apo-2 ligand / TRAIL or Fas ligand. The presence of the supplements of the invention may block such apoptotic activity and allow for improved expression of the protein of interest.

In addition, the methods can be used to increase the viability of cells undergoing freezing / storage/thawing procedures. During these procedures generally cells can lose viability. The presence of apoptosis inhibitors added to the cell culture media can provide for increased cell viability and aid in reducing or eliminating the variability in cell viabilities between aliquots or vials of cells.

IX. Kits

Also encompassed by the present invention are kits for promoting the viability of cells. In one embodiment a kit according to the present invention comprises: (a) one or more reagents or devices for transfection and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding using the transfection device, transfection agent and supplement.

In another embodiment a kit according to the present invention comprises: (a) one or more reagents or devices for freezing or thawing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for freezing or thawing cell lines and the use of the reagents.

In another embodiment a kit according to the present invention comprises: (a) one or more tissue culture products for culturing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for dilution cloning techniques and the use of the reagents in such approaches.

All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and indicated individually to be incorporated by reference in its entirety. The following examples illustrate but are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Expression of recombinant human lactoferrin.

A An Expression Vector For Human Lactoferrin Expression In Transgenic Rice

Complete nucleotide sequence of human mammary gland lactoferrin was codon optimized and synthesized by Operon Technologies (CA, USA). Human milk lactoferrin gene (Genbank accession number: HSU07642) was re-synthesized with codons most frequently used in translation of rice seed proteins in order to obtain optimal level of expression (~~Figure~~FIG. 1A-FIG. 1D). Although numbers of codons changed accounted for 22.46% of the entire sequence, amino acid composition remains identical to native human lactoferrin. The plasmid containing the codon-optimized gene was called Lac-ger. Lac-ger was digested with SmaI/XhoI and the fragment containing the lactoferrin gene was cloned into pAPI141 that was partially digested with NaeI and completely digested with XhoI. For expression of hLF in rice seeds, the codon-optimized gene was operably linked to the rice endosperm-specific glutelin (Gt1) promoter and NOS terminator. The resulting plasmid was designated pAPI164 (~~Figure~~FIG. 2).

B. Production system

Rice variety Taipei 309 (Oryza sativa, Japonica) was selected as the production system for recombinant human lactoferrin and transgenic rice events were generated by the particle bombardment of embryogenic rice calli with the plasmid pAPI164 and a companion marker plasmid containing the hygromycin phosphotransferase gene as a selectable marker. Fully developed, fertile rice plants were obtained by this procedure.

C. High Level Protein Expression of recombinant human lactoferrin in rice grain

Expression of recombinant human lactoferrin was under the control of the seed maturation-specific promoter Gt1. The high level expression of recombinant human lactoferrin is evident in ~~Figure~~FIG. 3 as independent transgenic rice events were screened. Total soluble proteins from mature rice seed extracts were run on Laemli gels and stained with Coomassie blue to visualize the proteins. An ~80kD recombinant lactoferrin protein was obtained in all transgenic lines as indicated by the stained gel. Expression levels of recombinant human lactoferrin corresponded to 0.5% of seed weight. The stable expression of recombinant human lactoferrin was monitored for 10 generations. The expression level is maintained at approximately 0.5% of seed weight of brown rice (~~Figure~~FIG. 4).

Example 2: Expression & purification of recombinant human transferrin.

Purification of recombinant human from rice grain.

The rice grain is dehusked and milled to an average of 100 mesh flour using standard food industry procedures Hulls and the solid material remaining after the extraction described below are destroyed Recombinant human transferrin is extracted from the flour with extraction buffer (20 mM Tris-Base, pH 7.5), with stirring, for 40 minutes at room temperature. Filter aid (Cellpure 300) is added at 20g/L to the extraction mixture before filtration through a filter press with 10 kDa regenerated cellulose.

The filter press filtrate was diafiltered and concentrated with a 30 KDa molecular weight cutoff cellulose membrane into10 mM Tris-Base pH. 8.5 to prepare for DEAE chromatography. The DEAE column was washed with (20mM Tris-Base, 40mM Sodium Chloride, pH 7 5) prior to loading the concentrate onto the column. After loading, the column was washed with 3 volumes of 10 mM Tris-Base, 0.5% (v/v) Triton X-114, pH 8.5 buffer followed by 7 volumes of 10 mM Tris-Base, pH 8.5 buffer. Transferrin was then eluded with 20mM Tris-Base, 40mM Sodium Chloride, pH 7.5.

Transferrin that was recovered from the DEAE column was diafiltered and concentrated with a 10 KDa molecular weight cut off cellulose membrane into 10 mM sodium phosphate, 25mM sodium chloride, pH 7 0 buffer. After diafiltration, recombinant transferrin was filtered though a 0.2 u filter prior to drying.

Example 3: Expression of recombinant human albumin in rice grain.

Protein sequences of human albumin (HSA) from various data bases were compared The consensus sequence represented by accession number P02768 (Swiss-Prot) was used as a base for gene codon-optimization for suitable expression of human albumin in rice grain (~~Figure~~FIG. 5A-FIG. 5D). Gene synthesis was carried out by Blue Heron (Seattle, WA) and the synthetic fragment was inserted into pUC based vector to create pUC-HSA. After confirmation of correct DNA and protein sequences, pUC-HSA was digested with MlyI and XhoI. The fragment containing codon-optimized HSA gene was inserted into pAPI405, which had been precut with NaeI and XhoI. Plasmid API405 was a derivative of pAPI141 which included Gt1 promoter, Gt1 signal sequence and a nos terminator.

Insertion of MlyI/XhoI fragment into pAPI405 resulted in pAPI504 (~~Figure~~FIG. 6). Plasmid API504 was then cleaved with HindIII and EcoRI. The HindIII/EcoRI fragment containing the entire expression cassette, Gt1 promoter, Gt1 signal sequence, codon-optimized HSA gene and

nos terminator, was cloned into pJH2600 predigested with the same enzyme resulting in pAPI508 (~~Figure~~FIG. 7). Plasmid JH2600 was a shuttle vector between E coli and Agrobacterium. After DNA sequence verification, pAPI508 was moved into Agrobacterium AGL-1 for rice transformation. Plasmid API504 was also used via bombardment-based transformation following the procedure described previously. Upon transformation, transgenic plants were generated and were sent to greenhouse where transgenic R0 plants grew to maturity and set R1 seeds.

To monitor the expression of HSA in rice seeds, 10 R1 seeds from each R0 plant were extracted using 10 mL of extraction buffer (50 mM Tris-HCl pH 8.0. 150 mM NaCl). The supernatant was collected and the expression level from rice extracts was monitored by an ELISA (Bethyl Laboratories, Montgomery, TX) The results showed that the HSA expression level in rice transgenic seeds ranges from 0.01 to 0.85% of brown flour weight (0.1 to 8.5 grams/kg flour). The results of the eight events with highest expression levels are shown in the following table (**Table E1**).

Table E1 Recombinant HSA expression level in top eight events

Line Number	Express Level (g/kg flour)
508-30	5.5
508-17	8.0
508-71	8.7
508-73	4.0
508-77	8.5
508-83	8.5
508-101	3.0
508-113	4.0

Example 4: Purification of recombinant human lactoferrin as cell culture media component.

To prepare cell culture media supplemented with recombinant human lactoferrin, recombinant human lactoferrin was purified from rice flour Transgenic rice line (164-12) expressing high levels of recombinant human LF was selected. This line, now named as LF164, was planted two generations per year, alternating field planting in summer and greenhouse planting in winter. For protein purification, paddy rice expressing rhLF was de-hulled (Rice Mill. PS-160, Rimac, FL), and then ground to flour (average particle size of 100 mesh) using a hammer mill

(8WA, Schutte-Buffalo, NY).

Protein extraction from transgenic flour was performed by mixing two kg of rice flour and 20 L of extraction buffer (0 02 M sodium phosphate pH 6.5 and 0.3 M sodium chloride) in a 50 L tank for 1 h. At the end of the mixing period, the suspension was allowed to settle overnight or centrifuged at 3750 rpm. In both cases, the supernatant was filtered through a plate and frame filter press (Ertel Alsop, 8S, NY) using M-05 and M-70 cellulose/perlite-based filters (Ertel Alsop, NY), respectively.

The filtrate containing rhLF and other rice flour soluble proteins was loaded onto an ion exchange column for further purification. An INDEX 200/500 process column (Amersham Pharmacia Biotech, NJ) packed with SP-Sepharose fast flow (Amersham Pharmacia Biotech, NJ) was used. The column was run at a linear flow rate of 150-200 cm/h. Packing, cleaning and testing of the resin bed was executed per manufacturer's instruction (HETP Test). The filtrate was loaded on to the resin at a linear velocity of 175 cm/h and washed with 0.02 M sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl until the A_{280} returned to baseline Recombinant hLF was eluted using 20 mM sodium phosphate buffer (pH 6.5) containing 0.8 M NaCl. The washing and elution were performed at 200 cm/h and 150 cm/h, respectively.

A Centramate module (Pall Biopharmaceutical, MA) with 1 ft^2 50 kDa polyethersulfone (Pall Biopharmaceutical, MA) membrane was used for concentration and desalting (ultrafiltration) of eluted hLF. The filtration was performed at a cross flow rate of approximately 1.5 L/min and an average transmembrane pressure of 10 psig. The eluted rhLF was concentrated and desalted to a final volume of 0.25 L and then lyophilized dry. Usually, about 3 grams of purified recombinant human lactoferrin was recovered from one kilogram of transgenic rice flour.

The recombinant human lactoferrin purified from rice flour is approximately 50% saturated with iron (partial-lactoferrin). The 50% saturated recombinant human lactoferrin was then made >90% iron saturated by iron up taking treatment, resulting in holo-lactoferrin and was made <10% iron saturated by acid treatment to remove bound iron resulting in apo-lactoferrin. The purified lactoferrins (holo-, partial- and apo-) were used as cell culture media components.

Example 5: Purification and characterization of recombinant serum human albumin (rHSA).

Overview of B000 production process.

To purify rHSA from rice grain, rice grain expressing recombinant human serum, as described above, was dehusked, ground to flour and mixed for 30 min. with rHSA extraction buffer (Sodium Acetate, pH 4.9). After clarification by filtration, the filtrate was loaded onto an anion-exchange column (Q-Sepharose; GE Healthcare) at a rate of approximately 150 cm/hr Once loaded, the resin was then washed with equilibration buffer to remove any unbound protein. The eluted rHSA is approximately 90% pure as analyzed by SDS-PAGE. Blue-Sepharose (GE Healthcare) can then be used to increase the purity from 90% to approximately 98% based on scanning analysis of SDS-PAGE gels. The rHSA is then concentrated and desalted via ultrafiltration prior to freeze drying.

Purified recombinant HSA was subjected to a range of biochemical and biophysical characterizations for comparison to pHSA (Plasma-derived HSA) (**Table E2**) These tests indicate that rHSA expressed in rice is equivalent to pHSA.

Table E2

Properties of recombinant albumin (rHSA) as compared to plasma derived albumin (pHSA)

Property	pHSA	rHSA
N-terminal sequence	DAHKSE	DAHKSE
Glycosylation	None	None
SDS-PAGE/Western	~66 kDa	~66 kDa
Molecular mass (MALDI)	66.9-68.1 kDa	66.8-67.9 kDa
Isoelectric focusing point	pI 5.3	pI 5.3
Ligand binding	Yes	Yes
Thermal stability	Midpoint 65°C	Midpoint 65°C
Esterase activity	Yes	Yes
Protease sensitivity	Same as each other	Same as each other

B0000C Production Process: For Ventria grown rice, the rice was harvested by combine or by hand. During this process the mature seeds were separated from the vegetative plant matter by the combine separator or by manual labor. The harvested rice was dried to approximately 12% moisture at which point it is suitable for storage in a clean grain bin, storage tote, supersack, or other container that will protect the grain from birds, rodents, lizards, insects and other pests. When the rice grain is needed for flour, it is first dehusked or dehulled. This process is done under vacuum such that debris and the outer part of the seed are swept away from the

endosperm and germ or bran layer. The dehusked grain is then either washed and dried, or washed and processed directly as in wet homogenization, or processed further in the dry, dehusked state. The dry, dehusked material may be debranned by a rice polishing or debranning machine which are common to white rice producers.

Debranned, dehusked rice may be washed at this point and wet-milled or dried for dry milling or processed directly by grinding into flour. Milling with the least amount of shear and heat is preferred as such with a roller mill or pin mill. A hammermill is also suitable. The flour should be ground such that the protein can be extracted to 90% in less than 5 minutes in water with hard agitation. Normally that requires a size of particle that is smaller than 400 micrometers or 4 mm. However, larger particles can be extracted if given longer time. Alternatively, the grain can be washed and wet milled with a liquid homogenizer set up such that 90% of the extractable protein is solubilized.

The flour slurry is typically mixed at a ratio of at least 3 parts water to 1 part flour and up to 20 parts water to 1 part flour. The water typically contains suitable buffers such as Tris/HCl, Citrate, Phosphate, HEPES, or the like, such that the pH is maintained around pH 7 and a small amount of salt such as 100 mM NaCl. After the slurry is homogenized in the case of wet milling, or mixed thoroughly for dry flour, the bulk solids are removed from the slurry by way of solid liquid separation. This is carried out by decanting, centrifugation, or filtration; for example using plate and frame with pads, pressure filter, belt filter, vacuum flask, hydroclone, or vacuum belt filter. After filtration, the compressed cake should be washed with extraction buffer to recover protein from the cake. The addition of diatomateous earth or other filter media is useful in promoting the clarity of the filtrate but is not necessary given the right equipment. Alternatively, a flocculating agent may be used to aid in clarification. The clarified filtrate should be checked for its albumin content and verified that the recovery is consistent with the determined expression level in the rice seed.

In order to remove starches, precipitable proteins, viruses, and other contaminants, 5 M acetic acid is added to the clarified filtrate until the pH reaches 5.0 and the solution turns white. The white solution is agitated for at least 20 minutes to encourage precipitation of insoluble materials. The precipitated solution is then filtered through a depth filter, such as a canister filter, cartridge filter or other filtration device to reach an optical clarity that is suitable for ultrafiltration, or less that 10 NTU (nephelometry turbidity units). It can also be clarified with a filter press, pressure filter, or alternatively by using a ceramic filter or other material that utilizes cross-flow. In addition, this material is suitable for direct application to an expanded bed

chromatography column.

In a preferred method, the clarified filtrate is clarified via filtration through a 0.2 micron filter, and neutralized to pH 7.0 with 1M NaOH. This material is then suitable for ultrafiltration by hollow fiber, flat sheet, or spiral wound cross flow filtration. The material can be passed through a membrane of 100 kilodalton (kDa) size or larger to remove viruses, unwanted larger contaminants, and aggregates. The material that passes through the membrane can be concentrated by a 10 or 30 kDa crossflow membrane and then the same membrane can be used to prepare the solution for chromatography. The concentrated material can then diafiltered with column equilibration buffer until the conductivity and the pH are equalized.
The preferred buffer for anion exchange chromatography on GE DEAE sepharose or GE Q sepharose is 10 or 20 mM Tris/HCl buffer pH balanced to pH 8.0. In contrast, the preferred buffer for cation exchange, for example via the use of for negatively charged resins or negatively charged resins mixed with a hydrophobic linker (mixed mode absorbents), or alternatively blue Cibicron such Blue Sepharose (GE) is acetate or citrate buffer pH balanced to 4.8 to 5.0

For either system the albumin and other similarly charged proteins will be retained by the matrix and washing is conducted to remove loosely bound material by washing with at least 5 column volumes of loading buffer, which may also include detergents as deemed necessary to help remove hydrophobic impurities. The material can be eluted by charging the column with the same or modified buffers with the pH increased 2-4 units for cation exchange or decreased 2-4 units for anion exchange. The resulting change in pH will allow for the exchange of ions and the protein will be eluted in a sharp band. To increase the purity of the elution fraction, the elution peak can be scrutinized such that the first portion (10%) or last (10%) or both portions can be excluded from the main elution peak. In the preferred method, a solution containing phosphate at 100 mM and pH adjusted to pH 4.0 including 10 mM NaCl is used to elute the protein from GE Q sepharose (Fast Flow). In this instance, pH and conductivity are used to elute the material allowing the discrimination between non-binding contaminants (flow through and wash) and tighter binding contaminants (those that are retained on the column in 100 mM Phosphate, 10 mM NaCl, and pH adjusted to 4.0)

After elution, if the pH of the eluted material has a pH of less than 6.0, then it is neutralized with 1M NaOH. The resulting solution is then diafiltered against the same buffer for the next chromatography step, which in a preferred method involves flowing the elutent through a column of the same matrix (i.e. Q sepharose) except in the non-binding mode with 100 mM

Phosphate, 10 mM NaCl, and pH 7.0.

The second column step uses the same principles as the first but in reverse mode such that the contaminants that were co-eluted on the binding column have an opportunity to be retained on the matrix at a neutral pH. The flow through material from the first capture column can also be treated with a variety of alternative types of chromatography approaches, for example, cation exchange, hydrophobic, mixed mode, or gel filtration chromatography.

In a preferred method, the flow through material from the Q sepharose non-binding column is concentrated on a 10 kDa or 30 kDa crossflow membrane until the concentration is between 15 and 25% albumin. The buffer is then changed by diafiltration into a suitable buffer for cell culture such as Dulbeccos PBS or alternatively 20 mM Phosphate, 50 mM NaCl, and pH 7.0. The material is then sterile grade filtered into a sterile container. The sterile filtered material may be treated with detergent to destroy enveloped viruses and to aid in the removal of hydrophobic toxins and contaminants. In a preferred method, 0.5% v/v Triton X-114 or X-100 is added to the 15 to 25% albumin solution at room temperature (less than 23 C and greater than 18 C) and the solution is agitated or stirred for at least hour. The material is then passed over a hydrophobic resin with a molecular weight exclusion limit that is much less than the molecular weight of albumin. Many commercially available resins are available including those from Biorad and Pall Corporation

The material that is passed over the column may then be tested in cells that are sensitive to detergent to confirm biological activity. The residual detergent that remains should typically be less than 0.005% with respect to the albumin solution. The detergent free flow through can then be sterile filtered into containers for direct shipment, or can have stabilizers added, or can be subjected to pasteurization with stabilizers, or can have stabilizers added before drying or dried directly. The material may be dried by lyophilization or spray drying. Prior to drying, in some instances, it may be useful to subject the material to a virus filtration step using a disposable, validated, virus removing capsule such as is available from GE, Pall, and Millipore. It is common in the art to understand that a prefiltration step may be necessary in order to effectively and economically pass the concentrated material through a 20 nm filter.

Results: Rice flour was extracted at 1:5 ratio in phosphate buffered saline and mixed for 20 minutes. The liquid was clarified using a Nalgene filter flask. The subsequent clarified extract was subjected to acid precipitation as is described in the methods. The solution was then filtered and neutralized to give a clarified filtrate. This material was diafiltered against 50 mM

Tris/Cl pH 8.0 until the material and buffer were equilibrated. The material was then loaded (300-600 cmh) on a pre-equilibrated GE Q-sepharose column to allow for 50 g/L binding capacity. The loaded material was washed with the same buffer and the material was then eluted with 100 mM Phosphate, 10 mM NaCl, and pH 4.0 as described above. The material eluted in a sharp peak and the collected eluate had a stable pH of about 5.8. Albumin produced using this method was compared to other sources of Albumin as more fully disclosed below: The eluate was collected in a pool and 1M NaOH was added until the pH was greater than 6.0. The material was then concentrated on a 10 kDa regenerated cellulose membrane approximately 5 fold and approximately five equal volume diafiltrations were carried out with 100 mM phosphate, 10 mM NaCl, pH 7.0. The final diafiltered material was checked for albumin protein content (in relation to the expression level in the starting material should be greater than 80%) and endotoxin level (should be less than 100 EU/mg depending on the feed material). This material was passed (60-160 cmh) over a Q-sepharose column, equilibrated with 100 mM phosphate, 10 mM NaCl, pH 7.0, of sufficient size to allow for approximately 2-3 times loading volume. The material was washed through the resin with the same buffer and collected. The collected material was diafiltered on a 10 kDa regenerated cellulose membrane and concentrated approximately 10 fold or until the albumin concentration reaches at least 10% or not more than 20% and five equal volume diafiltrations were performed with 20 mM phosphate. 50 mM NaCl, pH 7.0. After sterile grade filtration (0.2 µm), the solution was agitated for 1 hour with 0.5% (v/v) Triton X -100 at 20 +/- 2°C. After the incubation, the material was passed through Pall SDR resin according to the manufacturer's directions. The flow through material was sterile grade filtered into sterile containers and refrigerated or freeze dried as is common for protein and salt solutions.

Example 6: Comparison of recombinant albumin produced from rice using B0000C process compared to other sources of albumin and previous methods for the production of Albumin.

Methods: Albumin prepared using process B000C described in Example 5, was compared to albumin prepared using an alternative process (B000) which was previously used to prepare recombinant albumin (Cellastim (Batches B202 to B217))

Albumin production (old process, B000):

Rice flour and 25 mM Sodium phosphate, 50 mM Sodium Chloride, was pH balanced to 6.5 with NaOH and mixed for 20 minutes at room temperature with a S/L ratio of approximately 1:10. Filter aid (Cellpure 300) was added at 10 g/L and the slurry was filtered by filter press, vacuum

filtration, or centrifugation. The clarified filtrate was acid precipitated to about pH 5.0 with 1 M acetic acid. The resulting solution was filtered as described above with the addition of 5 g/L filter aid (Cellpure 300) The material was neutralized immediately to pH 6.5 to 7.0 with 1M NaOH. The material was diafiltered (10 kDa regenerated cellulose for all UFDF steps) with 5 equal diavolumes of the same buffer used for extraction. The material was loaded on a pre equilibrated Q-sepharose column (GE Healthcare) to allow for 8 g albumin binding per liter of resin at 60 cmh.

After washing the column with 5 column volumes of the same buffer, the albumin was eluted by increasing the salt concentration to 250 mM NaCl in one step. The resulting material was diafiltered against 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0 with 5-7 equal diavolumes. The resulting material was passed over a Q-sepharose column equilibrated with the 100 mM Sodium Phosphate, 10 mM NaCl, pH 7 0, and collected as flow-through The flow-through material was then concentrated and diafiltered against 20 mM sodium phosphate, 10 mM NaCl, pH 7.0 with 5 diavolumes The final concentrated material was sterile filtered and incubated with 10 g/L of the detergent CHAPS ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid) and mixed at room temperature for 1 hour. After the one hour incubation, the material was passed over a Biorad SM-2 column. The material was sterile filtered and freeze dried.
Size Exclusion Chromatography analysis.

Purity analysis by HPLC was carried out in 100 mM phosphate, pH 7.0 on a GF-250 column (Agilent Technologies) at a flow rate of 1 ml/min with the detector set at 214 and 280 nm. A standard curve was developed by injecting 5 different dilutions made by dry powder with a correction factor of 0 92 for salt and moisture. The main peak from 214 nm was integrated either by retention time or alternatively baseline. The unknown sample was injected at a concentration that is within the range of the standard injections. The unknown concentration of albumin per dry powder weight (purity) was calculated from the standard curve. In a typical experiment, the 0, 5, 8, 10, 15, and 20 µg of the standard was injected followed by approximately 10 µg of unknown sample in approximately 50 µL injection volume. The correlation coefficient for the standard curve after integrating the peaks was typically above 0.98.

SDS PAGE and Densitometry. (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)
Samples were prepared by diluting the protein solutions to 1-2 mg/ml to enable a defined amount of each protein to be loaded on to each well. The sample was mixed 1:1 with Tris-Glycine SDS sample buffer (LC2673 Novex) containing reducing agent (Invitrogen NP0004) and

heated to 70 °C for 5 minutes. The sample was loaded (10, 20, or 30 µg) onto a Novex 4-20% precast gel and separated at constant voltage (130V) in standard Tris-Glycine-SDS running buffer. The electrophoresis was ended when the tracking dye reached the end of the gel. A molecular weight marker was included in the first lane as a reference.

The gel was stained with G Bioscience (786-35G) and destained with water. A digital image was obtained with a Hewlett Packard Scanner (G4010). The image file was then opened with UN-SCAN-IT (Silk Scientific Corp.) The densitometry was carried out with positive image analysis in 256 grayscale in which all visible bands were included as individual segments. The background noise was corrected by four corner interpolation as specified in the software for each segment. The signal for each segment or band was then calculated from the product of the # of pixels and the average pixel intensity (0-255). The sum of the signals for an entire lane (all visible segments or bands) was taken as 100% and the impurity bands were subtracted to calculate the albumin purity. The percent of each contaminating protein in each band was calculated as the number of peptides identified for that contaminant protein as determined by peptide mapping divided by the total number of all peptides identified in a particular band. The image analysis was repeated 3 times such that the standard deviation is less than 0.5% out of 100%.

Determination of endotoxin by the Pyrogene rFC method

Endotoxin content was determined by the Pyrogene rFC method. Lyophilized endotoxin standard was mixed with endotoxin free water as specified by the manufacturer (Lonza) to develop a standard curve. The protein samples were either diluted as is for liquid or alternatively, reconstituted with endotoxin free water for powder. Different dilutions were prepared such that the readings should appear within the range of the standard curve. The samples were heated to 100 °C for 10 minutes to dissociate unwanted molecular interactions. In a typical experiment, the sample and standard were added at 100 µl per well, with 0, 0.001, 0.005, 0.01, 0.05, and 0.1 endotoxin units per well. The samples were also added at 100 µl and extra samples were included such that spiking with 0.001 -0.01 endotoxin units per well were added to test for assay inhibition or interference. The working reagent was prepared according to the manufacturer (Lonza) by mixing the rFC enzyme, assay buffer, and substrate in a 1:4:5 ratio, respectively. The working reagent was added to the wells at equal volume to the sample or standards. The fluorescence plate reader (Biotek FLX 800T) was set for excitation at 380 nm (bandwidth =20 nm) and the emission wavelength was set at 440 nm (bandwidth =30 nm). The reading taken at time zero is subtracted from the reading taken after 1 hour at 37 °C. The readings were considered valid if the correlation coefficient, slope, and Y-intercept for the

standards was within the set limits, and the spiking experiments show that the spiked endotoxin was measureable and recoverable within the set limits. In addition, the standard deviation for duplicate samples should be in reasonable agreement such that the standard deviation was within a specified arbitrarily chosen limit. All samples were collected aseptically and the tubes/vials/containers used for testing were verified to be extremely low endotoxin following good laboratory practices as they relate to accurate and precise endotoxin testing.

Determination of Cell Viability

The hybridoma cell line AE1 (ATCC) was maintained in DMEM basic media containing 5% fetal bovine serum (FBS). Albumin was tested under serum-free conditions (AFM6, KC Bio, Kansas) without supplementation of fetal bovine serum. The cells were subculture from 5% FBS to serum free media over multiple passages. At each subculture, the cells were analyzed for total cell count and viability in the presence of the indicated concentrations of albumin. (As assessed by trypsinization and direct counting using a Neubauer haemocytometer). The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Date show the number of viable cells / ml divided by 10^5

Determination of Detergent

The detergent concentration for the albumin was determined by a detergent (cell based) assay. Briefly, detergent sensitive cells were spiked with different amounts of detergent and the resulting cell viability cell determination used to generate a standard curve consisting of 16 independent data points. The change in viability with respect to the change in detergent concentration was plotted and fitted with a logarithmic function. This equation was then used to calculate the unknown detergent concentrations in samples tested in the same cell based assay The correlation coefficient for the standard curve for the data given was 0.9816 Typically detergent concentrations of greater than about 10 ppm per Cellastim dry weight, result in noticeable toxic activity. By comparison in a 10% albumin solution, toxic effects of detergent become apparent when the detergent concentration is above about 100 ppm to 200 ppm or 0.01% to 0.02 % (v/v).

Results & Discussion:

I. Analysis by Size Exclusion chromatography of plasma derived serum (Sigma Albumin), and recombinant HSA (Cellastim) produced using the new process (B000C) (Cellastim P0171) and the old process (Cellastim P0107)

The HPLC size exclusion profiles (~~Figure~~FIG. 8A, C & D) for the three types of albumin show that in terms of overall purity the different albumin preparations are generally similar. Specifically, the peaks at around 4.5 kDa and 240 kDa are the internal controls, while all three products contain a very small amount of an off main peak signal at about 10-12 kDa.

Human plasma derived albumin (Sigma Albumin) (~~Figure~~FIG. 8A), contains a contaminant at around 17 kDa, by comparison the recombinant rice derived albumin using the new process (Cellastim P0171) (~~Figure~~FIG. 8C) contains two protein contaminants of around 44 KDa and 55 kDa that occur in Cellastim made with the new process at significantly higher levels than when using the old process (Cellastim P0107) (~~Figure~~FIG. 8B) These peaks are not completely resolved in the HPLC separations, but can be seen as more clearly in the overlaid profiles of Cellastim P0171 and Sigma albumin (~~Figure~~FIG. 8D) and Cellastim made using the old and new processes shown in ~~Figure~~FIG. 8E.

The proteins corresponding to these peaks represent about 5% of all of the contaminant proteins identified by Peptide Mass Fingerprinting analysis of the main albumin peak in Cellastim produced using the process described in Example 5, as discussed further below.

All albumin products tested also contained a peak at around 130 kDa that most likely represents albumin dimers, it is noticeable that the Cellastim dimer peak is significantly smaller than the plasma derived albumin. The creation of aggregated albumin is an indicator of protein degradation which is used as one marker for degradation or loss of stability industry wide. It is likely that the Hsps present in Cellastim promote the disaggregation of the albumin, therefore reducing the number of dimers, since it is a commonly known function of Hsp 70 and other Hsp proteins.

II. Analysis by SDS PAGE of Cellastim batch **P0171** (New process) compared to albumin produced by Millipore/Novozymes (Cat No. 9301-01).

Results: SDS-PAGE analysis (~~Figure~~FIG. 9A & FIG. 9B) shows that in terms of overall purity the products are generally similar. ~~Figure~~FIG. 9A shows a comparison of Cellastim P0171 and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). ~~Figure~~FIG. 9B shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8) The six samples were loaded at 20 µg per lane.

Visual inspection of the gel shows that the new process which meets more rigorous specifications is more consistent among the 3 lots tested (~~Figure~~FIG. 9B, lane 2, 3, 4 vs lane 6, 7, 8). The banding pattern is significantly different among the three samples from the previous process as compared to the new process. Importantly, the new process samples have significantly less aggregates at around 250 KDa than the old process samples have. (Average greater than 2 % for the old process, and average less than 1 % for the new process). The identity of the protein contaminates was that are enriched in Cellastim produced using the new process is discussed further below.

III Analysis of endotoxin, detergent and growth promoting abilities of old and new batches of Cellastim A comparison of the performance of the two different processes for preparing several different lots of albumin (**Tables E3 and E4**) demonstrates that the old process produced recombinant albumin that contained significantly more endotoxin, and detergent compared to the new process described in Example 5, and resulted in a product that significantly enhanced cell viability.

Table E3

Cellastim - Old Process B000

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					1mg/ml	2 mg/ml	5 mg / ml	10 /mg ml
2/27/08	B202	70.6	25.3	815.3	15	7.9	4.1	0.9
2/27/08	B203	58.8	23.1	267.7	15.8	12.4	6.5	4.1
2/28/08	B204	59.9	29.2	589.2	16.3	11.1	5 2	2.9
2/28/08	B205	63	64.3	133.4	14	11.6	6.9	3.8
3/3/08	B206	41.1	35.1	1182.2	14 9	2.3	0.0	0.0
3/3/08	B207	94.2	28.7	388	16.5	12.7	7.6	4
3/4/08	B208	24.5	>80	467 2	15.3	11.4	7 1	2.7
3/4/08	B209	66.1	91.8	406.4	16.7	15.2	6.9	4.1
3/4/08	B210	87.3	67.8	76.4	17 5	19.1	15.4	8.5
3/11/08	B217	62.09	4.4	127.3	16.7	18.7	13.2	6.6
Averages			**44.97**	**445.31**	**15.87**	**12.24**	**7.29**	**3.76**

Table E4								
Cellastim - New Process B0000C								
Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					0.5mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/2/2009	B0032C	209.4	0.35	3.1	13.1	15.7	13.4	11.2
2/2/2009	B0033C	271.4	0.26	7.9	18.4	17.4	15.1	14.3
2/10/2009	B0041C	247.1	0.18	5.4	15.4	17.2	14.1	12.1
4/27/2009	B0118C	617.4	0.11	Less than 1	15.7	15.4	19.2	16.8
5/14/2009	B0138C	610.6	0.48	Less than 1	12.5	15.8	15.1	14.6
6/9/2009	B0158C	598.0	0.13	3.9	13.7	17.1	13.2	11
6/15/2009	B0162C	618.6	0.20	2.0	15.5	17.5	17.2	14.7
7/28/2009	B0196C	507.0	0.36	3.9	17.4	17.3	18.8	14.8
8/26/2009	B0219C	851.4	0.86	8.2	17.9	16.3	16.6	13.7
8/31/2009	B0220C	897.8	0.93	Less than 1	13.5	16	15.5	15.1
9/9/2009	B0227C	929.9	0.13	Less than 1	11.1	14.4	12.5	11.4
Averages			0.36	3.45	14.92	16.37	15.52	13.6

Discussion: Re-engineering the old process to create the new process described in Example 5 resulted in significant changes in both overall product purity, and performance, as described more fully below.

The changes made it possible to make products that were lower in detergent, lower in endotoxin, and increased purity. Specifically, the new process routinely produced recombinant albumin with an overall purity of greater than about 95% By comparison the old method routinely produced albumin with a maximum purity of about 90 %. Surprisingly, despite the increased product purity, these changes in processing also resulted in enhanced co-purification of heat shock proteins, (see below) with the recombinant albumin. Without being bound by any particular theory of operation, it is believed that the combination of high albumin purity, relative lack of endotoxin and / or detergent, and co-purification of heat shock proteins results in a product that significantly out performs previous methods for preparing albumin.

Specifically **Tables E3** and **E4** demonstrate that the new process for producing recombinant albumin results in a product that, for example at 5 mg /ml, results in an average batch to batch

100 percent improvement in cell viability (at 5 mg /ml), and also results in a product with an average 100-fold less endotoxin, and 100 fold less detergent than the old process.

Example 7: Analysis of the effects on cell growth and viability.

To compare the cell growth promoting abilities of the recombinant albumin prepared above, to other commercially available albumin products, the different sources of albumin they were compared side by side in a cell growth and viability assay. The three products tested were (Cellastim, Lot # P0153) Cellprime albumin (Millipore/Novozymes Cat No. #9301-01), and plasma derived albumin (Seracare Cat No. #HS-400-60).

Methods: Specially conditioned Hybridoma cells AE1 were seeded in DF12/ITSE at a density of 0.5 x 10^5 cells per ml of media after washing twice with same media to remove residual media. The media and cells were then left untreated (negative control), treated with Seracare albumin, treated with Cellprime albumin, and treated with rice derived recombinant albumin at the concentrations shown in the figure legend. The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Results are shown in ~~Figure~~FIG. 10A-FIG. 10C.

Results: Novazyme's Cellprime albumin caused a loss in viability (cross-hatch bars). Seracare albumin (white bars) caused a measureable increase in viability but not as large an increase as is seen with recombinant albumin made in rice with the new process (Cellastim) (black bars). Under these conditions the rice derived recombinant albumin was approximately 5 times as active in promoting cell viability compared to any of the other albumin products, at any concentration tested. The negative control is represented by the striped bars.

Discussion: Given the possibility that the other commercially available albumin products may have similar overall purity, endotoxin and detergent levels to rice derived recombinant albumin, the dramatically superior performance of the recombinant rice derived albumin compared to other commercially available albumins suggests that the previously un-identified protein contaminants identified in Cellastim compared to the plasma derived albumin (Example 5) could be having a positive impact on cell viability. To identify and then characterize the impact of these proteins on the properties of Cellastim, a sample of the recombinant albumin was subjected to peptide mass finger printing, as described below

Example 8: Peptide Mass Finger printing of recombinant human serum albumin.

Methods: Samples of albumin were analyzed to determine significant protein contaminants using a NanoLCMS/MS peptide sequencing system (ProtTech, Inc), and proprietary software to identify the proteins based on the molecular weight of the peptide fragments. In brief, samples of albumin were analyzed by SDS-PAGE, and each major band gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin. The resulting peptide mixture was analyzed by a LC-MS/MS system, in which a high pressure liquid chromatography (HPLC) with a 75 micrometer inner diameter reverse phase C18 column was used in-line coupled with an ion trap mass spectrometer. The mass spectrometric data acquired was used to search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting.

Results: Upon testing of three representative lots of recombinant albumin, three Hsp70 proteins were identified by Peptide Mass Fingerprinting (**Table E5**). The three specific sequences identified: ABF95267, ABA97211, and BAD 07938 were compared to the non redundant database to identify highly related and homologous proteins. The results of the top hits from each of these comparisons is shown in **Tables E6, E7 and E8** below.

Table E5

Peptides identified from Cellastim by mass finger printing

Sequence	Peptide
ABF95267	ATAGDTHLGGEDFDNRVVPGPADKSPMIVVTYKGEEK NAVITVPAYFN DSQRIINEPTAAAIAYGLDKK **(SEQ. ID. No. 2)**
AAB63469 BAD07938 BAD07713	NQAAVNPER NGHVEIIANDQGNRIVNKDGKPYIQVK IINEPTAAAIAYGLDKK KLGTVIGIDLGTTYSCVGVYK VEIESLFDGTDSFSEPLTR **(SEQ. ID. No. 3)**
ABA97211	NQADSVVYQTEK KQDITITGASTLPKDEVERDVVLLDVTPLSLSLGLET LGGVMTK **(SEQ. ID. No. 4)**

Results of sequence comparisons to ABF95267 sequences in the non redundant database of protein sequences in Genbank® (Nucleic Acids Research, 2008 Jan;36(Database issue).D25-30) are shown in **Table E6** below:

Table E6

Sequences producing significant alignments with ABF95267:

Gene Refs	Gene description	(Bits)	Value
ref|NP_001140835.1|	hypothetical protein LOC100272911 [Zea ma...	79.7	8e-14
ref|XP_002465468.1|	hypothetical protein SORBIDRAFT_01g039390...	79.7	9e-14
ref|NP_001049719.1|	**Os03g0277300 [Oryza sativa (japonica cult...**	**79.7**	**9e-14**
gb|ACJ54890.1|	heat shock protein 70 [Oryza sativa Japonica G...	79.7	9e-14
Sp|P09189.1|	HSP7C_PETHY RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
emb|CAA31663.1|	hsp70 (AA 6 -- 651) [Petunia x hybrida]	77.0	5e-13
ref|XP_002312089.1|	predicted protein [Populus trichocarpa] >...	77.0	5e-13
sp|P24629.1|	HSP71_SOLLC RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
gb|AAB99745.1|	HSP70 [Triticum aestivum]	76.6	6e-13
gb|AAB42159.1|	Hsc70 [Lycopersicon esculentum]	76.6	7e-13
gb|ACD45076.1|	heat-shock protein 70 [Dactylis glomerata]	76.3	8e-13
ref|XP_002512741.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
ref|XP_002512742.1|	heat shock protein, putative [Ricinus com...	75.9	1e-12
gb|AAA82975.1|	PsHSP71.2 >emb|CAA67867.1| heat shock protein ...	75.9	1e-12
gb|AAS09825.1|	heat shock cognate protein 70 [Thellungiella h...	75.9	1e-12
emb|CAA44820.1|	heat shock protein 70 [Nicotiana tabacum]	75.5	2e-12
ref|NP_001055754.1|	Os05g0460000 [Oryza sativa (japonica cult...	75.5	2e-12
ref|NP_001051724.1|	Os03g0821100 [Oryza sativa (japonica cult...	75.1	2e-12
ref|XP_002456611.1|	hypothetical protein SORBIDRAFT_03g039360...	75.1	2e-12
ref|NP_001044757.1|	Os01g0840100 [Oryza sativa (japonica cult...	75.1	2e-12
gb|ACR35910.1|	unknown [Zea mays]	75.1	2e-12
ref|XP_002284017.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002532297.1|	heat shock protein, putative [Ricinus com...	75.1	2e-12
ref|XP_002284008.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002283532.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.7	3e-12
ref|XP_002332067.1|	predicted protein [Populus trichocarpa	74.7	3e-12
gb|AAF34134.1|	high molecular weight heat shock protein [Malu...	74.7	3e-12
gb|EEC76425.1|	hypothetical protein Osl_14101 [Oryza sativa I...	74.7	3e-12
ref|XP_002316294.1|	predicted protein [Populus trichocarpa] >...	74.7	3e-12
ref|XP_002283516.1|	PREDICTED: similar to HSC70-1 (heat shock...	74.3	3e-12
ref|XP_002441219.1|	hypothetical protein SORBIDRAFT_09g022580...	74.3	4e-12

Results of sequence comparisons of ABB63469 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E7** below:

Table E7

Sequences producing significant alignments with AAB63469:

Gene Refs	Gene description	(Bits)	Value
emb|CAP31983.1|	C. briggsae CBR-HSP-4 protein [Caenorhabditis...	50.1	7e-05
dbj|BAG60366.1|	unnamed protein product [Homo sapiens]	49.7	8e-05
ref|YP_002421952.1|	chaperone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|YP_001640420.1|	chaperone protein DnaK [Methylobacterium ...	49.7	9e-05
ref|NP_001105893.1|	Binding protein homolog1 precursor [Zea m...	49.3	1e-04
gb|AAA62325.1|	HSP70	49.3	1e-04
ref|YP_001756576.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
gb|AAB63469.1|	endosperm luminal binding protein [Oryza sativa]	49.3	1e-04
ref|YP_001925829.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
ref|NP_001105894.1|	Binding protein homolog2 precursor [Zea m...	49.3	1e-04
gb|ACF86491.1|	unknown [Zea mays]	49.3	1e-04
ref|NP_001045675.1|	**Os02g0115900 [Oryza sativa (japonica cult...**	49.3	1e-04
ref|ZP_02191025.1|	Molecular chaperone (alpha proteobacterium...	49.3	1e-04
ref|XP_001701685.1|	binding protein 1 [Chlamydomonas reinhard...	48.5	2e-04
ref|XP_001701884.1|	binding protein 2 [Chlamydomonas reinhard...	48.5	2e-04
emb|CAC37635.1|	luminal binding protein, BiP [Scherffelia dubia]	48.1	3e-04
gb|AAM93256.1|	heat shock protein 70-C [Heterodera glycines] ...	48.1	3e-04

Results of sequence comparisons of sequence ABA97211 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E8** below:

Table E8

Sequences producing significant alignments with ABA97211

Gene Refs	Gene description	(Bits)	Value
gb|AAK13022.1|	heat shock protein 70 [Fibrobacter succinogene...	44.3	0.004
ref|XP_002442079.1|	hypothetical protein SORBIDRAFT_08g009580...	44.3	0.004
gb|EEC69073.1|	**hypothetical protein OsI_37938 [Oryza sativa I...**	**44.3**	**0.004**
ref|XP_001752769.1|	predicted protein [Physcomitrella patens ...	44.3	0.004
ref|NP_001066486.1|	Os12g0244100 [Oryza sativa (japonica cult...	44.3	0.004
gb|ACT65562.1|	70 kDa heat shock protein [Triticum aestivum]	43.9	0.005
ref|NP_001152528.1|	stromal 70 kDa heat shock-related protein...	43.9	0.005
gb|ACN31310.1|	unknown [Zea mays]	43.9	0.005
ref|XP_001772650.1|	predicted protein [Physcomitrella patens ...	43.9	0.005
ref|NP_001146752.1|	hypothetical protein LOC100280354 [Zea ma...	43.9	0.005

gb|ABP65327.1|	chloroplast heat shock protein 70 [Pennisetum ...	43.9	0.005
gb|AAO72585.1|	heat shock-related protein [Oryza sativa (japo...	43.5	0.007
ref|YP_001740846.1|	Chaperone protein dnaK (Heat shock protei...	43.5	0.008
ref|ZP_03728467.1|	chaperone protein DnaK [Dethiobacter alkal...	43.1	0.009

Discussion: Peptide Mass Fingerprinting identified 3 rice heat shock protein super family members that co-purify with albumin, 2 Rice HSP70 genes, (gb|ACJ54890.1|), EEC69073, and AAB63469 - a BiP homolog from rice endosperm tissue (endosperm lumenal binding protein). The complete amino acid sequences coded by these genes are listed below:

Gene gb|ACJ54890.1| heat shock protein 70 [Oryza sativa Japonica Group] HSP70 was found to occur in recombinant albumin in Cellastim at approximately 0.07% wt / wt. Its complete amino acid coding sequence (**SEQ. ID. No. 5**) is provided below:

MAGNKGEGPA IGIDLGTTYS CVGVWQHDRV EIIANDQGNR TTPSYVAFTD TERLIGDAAK
NQVAMNPTNT VFDAKRLIGR RFSDPSVQAD MKMWPFKVVP GPADKPMIVV TYKGEEKKFS
121 AEEISSMVLT KMKEIAEAFL STTIKNAVIT VPAYFNDSQR QATKDAGVIS GLNVMRIINE
181 PTAAAIAYGL DKKAASTGEK NVLIFDLGGG TFDVSILTIE EGIFEVKATA GDTHLGGEDF
241 DNRMVNHFVQ EFKRKHKKDI TGNPRALRRL RTACERAKRT LSSTAQTTIE IESLYEGIDF
301 YATITRARFE ELNMDLFRRC MEPVEKCLRD AKMDKAQIHD VVLVGGSTRI PKVQQLLQDF
361 FNGKELCKSI NPDEAVAYGA AVQAAILSGE GNQRVQDLLL LDVTPLSLGL ETAGGVMTVL
421 IPRNTTIPTK KEQVFSTYSD NQPGVLIQVY EGERTRTKDN NLLGKFELTG IPPAPRGVPQ
481 INVTFDIDAN GILNVSAEDK TTGKKNKITI TNDKGRLSKE EIERMVQEAE KYKAEDEQVR
541 HKVEARNALE NYAYNMRNTV RDEKIASKLP ADDKKKIEDA IEDAIKWLDG NQLAEADEFE
601 DKMKELESLC NPIISKMYQG GAGGPAGMDE DAPNGSAGTG GGSGAGPKIE EVD

AAB63469 BiP homolog from rice endosperm tissue (endosperm lumenal binding protein [Oryza sativa]) BiP was found to occur in recombinant albumin in Cellastim at about 0.09% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 6**) is provided below:

mdrvrgsafl lgvllagslf afsvakeetk klgtvigidl gttyscvgvy knghveiian
dqgnritpsw vaftdserli geaaknqaav npertifdvk rdigrkfeek evqrdmklvp
121 ykivnkigkp yiqvkikdge nkvfspeevs amilgkmket aeaylgkkin davvtvpayf
181 ndaqrqatkd agviaglnva riineptaaa iaygldkkgg eknilvfdlg ggtfdvsilt
241 idngvfevla tngdthlgge dfdqrimeyf iklikkkysk diskdnralg klrreaerak
301 ralsnqhqvr veieslfdgt dfsepltrar feelnndlfr ktmgpvkkam ddagleksqi

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361 heivlvggst ripkvqqilr dyfegkepnk gvnpdeavay gaavqgsils geggdetkdi
421 lildvapltl gietvggvmt kliprntvip tkksqvftty qdqqttvsiq vfegersmtk
481 dcrllgkfdl sgipaaprgt pqievtfevd angilnvkae dkgtgkseki titnekgrls
541 qeeidrmvre aeefaeedkk vkeridarnq letyvynmkn tvgdkdklad kleseekekv
601 eealkealew ldenqtaeke eyeeklkeve avcnpiisav yqrtggapgg rrrgrlddeh
661 del
```

EEC69073 / Osi_37938 [Oryza sativa Indica Group] The stromal HSP70 was found to occur in recombinant albumin in Cellastim at about 0.06% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 7**) is provided below:

```
masftsqlga macgaapsts plaarrsgql fvgrkpaaas vqmrvpragr argvamrvac
61 ekvvgidlgt tnsavaameg gkptvitnae ggrttpsvva ytkggerlvg qiakrqavvn
121 pentffsvkr figrkmaevd deakqvsyhv vrddngnvkl dcpaigkqfa aeeisaqvlr
181 klvddaskfl ndkitkavvt vpayfndsqr tatkdagria glevlriine ptaaslaygf
241 ekknnetilv fdlgggtfdv svlevgdgvf evlstsgdth lggddfdkfy fcwvfyfgam
301 thetpkvvdw lasnfkkdeg idllkdkqal qrlteaaeka kmelstlsqt nislpfitat
361 adgpkhiett lsrakfeelc sdlidrlktp vtnalrdakl svdnldevil vggstripsv
421 qelvkkitgk dpnvtvnpde vvslgaavqg gvlagdvkdv vlldvtplsl gletlggvmt
481 kiiprnttlp tsksevfsta adgqtsvein vlqgerefvr dnkslgsfrl dgippaprgv
541 pqievkfdid angilsvaai dkgtgkkqdi titgastlpk devermveea dkfaqedkek
601 rdaidtknqa dsvvyqtekq lkelgdkvpa pvkekvdaki nelkealagg stqsmkdama
661 alneevmqig qamynqqpna gaagptpgad agptssggkg pndgdvidad ftdsn
```

Because these proteins only occur at low levels in the new batches of rice recombinant albumin relative to albumin, a pre-requisite for confirming that these contaminants are actually responsible for the superior growth promoting effects of the new batches of recombinant albumin is to determine whether the addition back of these components to albumin restores or enhances the growth promoting activities of the recombinant albumin at levels which are comparable to those actually identified for each component in Cellastim.

Example 9: Separation of heat shock proteins from recombinant albumin by affinity chromatography.

Methods: Cellastim produced using the new process [Lots P0153, P0156, and or P0171] powder was mixed with purified water at approximately 20 g/L. The resulting solution was diafiltered against 50 mM Tris/Cl, pH 7.0 with at least 5 equal volumes of buffer. The resulting solution was passed over an ATP agarose column and the resulting flow through was labeled as fraction A. The column was washed with 5 column volumes of the equilibration buffer and the

material bound to the ATP-agarose was eluted with 50 mM Tris/Cl, 1M KCl, pH7.0. The eluted material was labeled as fraction B. The wash was kept as fraction C. Fraction A was directly concentrated to 100 g/L and diafiltered with d-PBS. Fraction B was concentrated significantly, up to 20 fold or 100 fold in 50 mM Tris/Cl for further analysis. The wash fraction C was kept for further reference. For Western blotting, 10 µg of each protein fraction (by A280, where the e.c. (extinction coefficient) of albumin is 0.53 cm^2/mg and e.c. of Hsp70 is 0.41 cm^2/mg) were loaded on a 4-20% SDS PAGE gel in 2X SDS loading buffer. The samples were heated to 80 °C for approximately 5 minutes before loading. The separation was done at 200V (constant voltage) and ran for approximately 90 minutes. The resulting gel was rinsed in water for 30 minutes to 2 hours and then the proteins were transferred to a Nitrocellulose membrane at 30 mA (constant current) for 2 hours. The resulting blot contained the molecular weight marker proteins as a transfer control and was then blocked in 5% (w/v) milk powder in water. The primary monoclonal antibody (a mouse anti-bovine Hsp70(Sigma/Aldrich #H5147)) was added in 5% milk solution to the blot (1:2500) and the blot was incubated on a rocker with gentle rocking overnight at 4 °C. The blot was then washed 4 times for 10 minutes each in TDN and the secondary antibody (Pierce anti-mouse HRP conjugated) in 5% milk solution which was added at a dilution of 1:2500. After incubation at 4 °C for 2 to 3 hours, the blot was washed 4 times with TDN for 10 minutes each. The resulting blot was then incubated with pico (Pierce) chemiluminescent substrate for 5 minutes. Kodak photographic film was exposed to the blot in a dark room and the subsequent film was developed, rinsed, fixed, rinsed, and dried. To determine accurate transfer of the molecular weight marker position onto the film, a light emitting label was used.

Results: The results are shown in ~~Figure~~FIG. **11**. The Western blot pictured shows that the separation scheme produces two populations of proteins in the A (flow through) and B (ATP binding) fractions. The starting material, (lane 2) the fraction A flow through, (lane 3) fraction C wash, (lane 4) and fraction B (lane 5) were tested for the ability to react to the monoclonal antibody. In addition, a commercially available Hsp70 protein that serves as a positive control was loaded in the last lane (lane 10). As shown in the blot in ~~Figure~~FIG. **11**, the flow through fraction A (lane 3) does not contain significant amounts of Hsp70. The eluted and concentrated fraction B (lane 4) is highly reactive to the antibody as shown in the blot and indicates at least two distinct bands centered around the 75 kDa molecular weight marker. The wash fraction C (lane 5), indicates the presence of two bands that run at slightly below 75 kDa. In a separate independent experiment, the flow through fraction A (lane 7) again is not reactive to the antibody, and the wash fraction C (lane 8) is also not reactive to the antibody, but the fraction enriched in ATP binding proteins (Fraction B) shown in lane 9 gives the same banding pattern as was seen from the first separation.

Discussion: A separation protocol was developed to separate HSP70 proteins based on their ability to bind to ATP agarose affinity resin, and. was tested for its effectiveness. The procedure involved only minimal sample manipulation, using only ATP agarose, and ultrafiltration to concentrate and conduct buffer changes, and an anti-hsp70 antibody to detect the presence of hsps. The results of the procedure (~~Figure~~FIG. 11) clearly demonstrates that while in 10 µg of starting material, 10 µg of flow through, or 10 µg of wash fraction there is insufficient hsp70 to be detected by the ant-Hsp antibody By contrast, the fraction eluted from the ATP agarose column, contains at least two proteins that are clearly recognized by the anti-Hsp70 antibody The results therefore show that the separation scheme was successful and reproducible on two independent chromatography runs and diafiltrations. Furthermore, the data substantiates the identification of heat shock proteins made by Peptide Mass Finger printing, as discussed earlier, and demonstrates that these proteins are functional and can be readily isolated and enriched by simple ATP agarose chromatography followed with diafiltration. It is concluded that the heat shock proteins co-purify with the recombinant albumin. Such co-purification is consistent with the hypothesis that the heat shock proteins are bound to the albumin, and that the albumin acts to stabilize the heat shock proteins in a stable conformation. Surprisingly, the recombinant albumin / heat shock protein complex retains significant ATPase activity (data not shown) consistent with the presence of function heat shock proteins. This increased activity was further confirmed as providing a growth promoting effect as described below.

Example 10: Impact of the removal of Hsps from rice recombinant albumin on cell viability.

Methods: The separation scheme described in Example 9 was also used to produce fraction A suitable for cell culture testing (~~Figure~~FIG. 12). The method involves minimal manipulation of fraction A, as it is flowed through an ATP agarose column and then concentrated by diafiltration and buffered with PBS that is suitable for cell culture. The intent of the method is to not introduce new variables into the experiment such that a loss of viability is seen but due to some other reason or cause beyond the removal of ATP binding proteins. Fraction A was tested against the unadulterated control (starting material) for ability to promote hybridoma cell culture viability. The results of the test are shown in ~~Figure~~FIG. 12.

Results: As shown in ~~Figure~~FIG. 12 the Cellastim starting material (cross hatched bars), and Part A (solid bars) were tested at the same concentration and compared to the negative control (striped bars). A statistically significant decrease is observable at all four concentrations tested The result indicates that there was a significant loss in the performance of Cellastim after ATP agarose treatment. The treatment resulted in a 28.0, 21.7, 26.7, and 79.5 % loss as compared to Cellastim before removal of ATP binding proteins. In this experiment, care was taken in the

design and handling of the samples to ensure that any inadvertent losses in performance due to sample handling or the accidental introduction of new contaminants were minimized.

Discussion: The cell culture results (~~Figure~~FIG. 12) demonstrate that it is possible to reduce the performance of the rice derived recombinant albumin by simply passing it over an ATP binding column. This data, when combined with the results shown in Example 9 demonstrates that the depletion of the hsps from albumin by the ATP agarose column directly reduces the cell growth promoting properties of the albumin. This result therefore demonstrates that the superior properties of the albumin arise, at least in part, from the contaminating heat shock proteins in the albumin.

Example 11: Effect of rice derived recombinant human serum albumin in combination with lactoferrin on the proliferation of CHO K1 in serum free culture.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components when added in combination with these components, a series of additional experiments were designed to evaluate and identify optimum combinations of such components.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 μM methotrexate, 50 μg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 μg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or rLF or various mixtures of the two additives. Control cultures without added rHSA or rLF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. All cultures were grown at 37°C, 6% CO_2 in a humidified environment. The Observed Effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Table E9 shows the number of viable cells after 6 days of culture Cells in media without supplementation proliferated to 3.3 x 10^5 viable cells/ml. Addition of plant derived recombinant human serum albumin (rHSA) or recombinant lactoferrin (rLF), or a combination of the two increased the proliferation of cells as indicated below. Column two shows the Observed Effect of the respective additive(s) above baseline control culture. Column 3 shows the Predicted Effect using a model where the predictive additive effect equals the sum of additive1 and additive2.

TABLE E9				
	Viable cells(e^5)/ml	**Observed Effect**	**Predicted Effect**	**Ratio of Lactoferrin to albumin**
No Additives (Baseline)	3.3	0		
rHSA 0.5 g/L	7.3	4.0		
rHSA 1 g/L	7.9	4.6		
rLF 0.1 g/L	7.5	4.2		
rLF 0.8 g/L	16.0	12.7		
rLF 0.1 g/L +rHSA 0.5 g/L	18.9	15.6	8.2	1 : 5
rLF 0.8 g/L +rHSA 0.5 g/L	29.0	25.7	16.7	1 : 0.625
rLF 0.1g/L +rHSA 1g/L	23.6	20.3	8.8	1 : 10
rLF 0.8g/L +rHSA 1g/L	35.4	**32.1**	17.3	. 1.25

Discussion: Lactoferrin is an essential factor for serum free cell culture media, where it acts to transport iron, additionally Lactoferrin has more recently been identified as a growth factor and inhibitor of apoptosis in some cell types (Huang et al., In vitro Cell. Dev. Biol. Anim. (2008) 44(10) 464-71; Wong et al., Rheumatology 48(1) 39-44; Bi et al., Arch. Immunol. Ther. Exp. (1997)45(4) 315-20,Hashizume et al., Biochem. Biophys. Acta. (1983) 763(4) 377-82). In **Table E9**, the data shows that the addition of 0.1 g/L and 0.8 g/L recombinant lactoferrin resulted in a significant enhancement of cell growth compared to the baseline rate of cell growth consistent with lactoferrins growth stimulating properties.

The growth stimulating effect of lactoferrin was further enhanced by the addition of plant derived recombinant human serum albumin. Unexpectedly the combination of 0.1 g/L lactoferrin and 0.5 g/L plant derived recombinant human serum albumin (1 to five ratio) resulted in a stimulation in cell growth that was approximately 4 fold higher than either albumin alone or lactoferrin alone

tested at the same concentrations. While the combination of 0.1 g/L lactoferrin and 1.0 g/L human serum albumin (1 to 10 ratio) resulted in a stimulation in cell growth that was approximately 4.4 to almost 5 fold higher than either albumin or lactoferrin alone at the same concentrations.

Synergism was also observed when plant derived recombinant human serum albumin was added to the lactoferrin at a concentration of 0.8 g/L. Even at this significantly higher concentration, the addition of plant derived recombinant human serum albumin further increased cell growth by 2 to 2.5 fold

Albumin and lactoferrin are distinct protein families with little overlapping structural or functional similarity. Thus the fact the synergism was observed with these proteins is unexpected based on their dissimilar structure & function. Synergism provides the opportunity to develop improved supplements that provide for supplements with enhanced cell culturing properties (i.e. improved cell growth and viability) by using optimal ratios of lactoferrin and albumin.

Example 12: Effect of rice derived recombinant albumin in combination with lactoferrin on the productivity of CHO K1 cells grown in serum free culture

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of plant derived recombinant human serum albumin or LF or various mixtures of the two additives. Control cultures without added HSA or LF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. Antibody productivity was determined at the end of the batch culture when cell viability had declined to <10% (typically day 13 or 14) by quantitative ELISA (Bethyl Laboratories). All cultures were grown at 37°C, 6% CO_2, in a humidified environment. The

Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

TABLE E10				
	Product Conc. (µg/ml)	**Observed Effect**	**Predicted Effect**	**Ratio of HSA to Lactoferrin**
No Additives (Baseline)	47.1	0		
rHSA 0.5g/L	114.6	67.5		
rHSA 1g/L	136.1	89.0		
rLF 0.1g/L	91.9	44.8		
rLF 0.8 g/L	138.5	91.4		
rLF 0.1g/L + rHSA 0.5g/L	183.7	136.6	112.3	1 : 5
rLF 0.8g/L + rHSA 0.5g/L	242.1	195.0	158.9	1 : 0.625
rLF 0.1g/L + rHSA 1g/L	219.3	172.2	133 8	1 : 10
rLF 0.8g/L + rHSA 1g/L	260.2	213.1	180 4	1. 1 25

Table E10 shows the effect of plant derived recombinant human serum albumin and recombinant lactoferrin produced in monocot cells, and various combinations of the two proteins on the productivity of CHO K1 cells. Table E10 shows that the concentration of antibody product produced by the cells after 13 days of culture in the baseline medium (with no additives) produced 47.1 µg/ml of antibody product. Medium supplemented with recombinant albumin or recombinant lactoferrin alone or a combination of plant derived recombinant human serum albumin and lactoferrin produced significantly higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the predictive additive effect of the combination of two additives using a model where the predictive additive equals the sum of each additive alone at the same concentration.

Discussion: Table E10 demonstrates that improvements in cell growth and viability directly translated into improved antibody expression and cell productivity. As was the case for cell viability and cell growth, maximal effects of the protein components was observed when these were added to the culture together.

Table E11 shows an additional data set showing the effect of the additives at an expanded range of concentrations, using the same conditions as in **Example12.** Medium supplemented with recombinant albumin alone, or lactoferrin alone, or a combination of both proteins, produced higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the Predicted Effect of the combination of two additives using a model where the Predicted Effect equals the sum of the effects of each additive when added alone at the same concentration. The data indicates that the actual effect of the combination is greater than the Predicted Effect over all of the ratios of the two proteins tested.

TABLE E11

	Product Conc. (ug/ml)	Observed Effect	Predicted Effect	% Improvement over predicted activity	Ratio of Lactoferrin to HSA
No additives (Baseline)	56.9	0.0			
Single additives					
HSA 0.5g/L	99.1	42 2			
HSA 1 g/L	126.3	69.4			
HSA 2 g/L	186.0	129.1			
HSA 3 g/L	212.9	156.0			
LF 0.1g/L	123.1	66.2			
LF 0.5g/L	173.5	116.6			
LF 0.8g/L	182.0	125.1			
LF 1.5g/L	196.3	139.4			
Combinations					
LF 0.1g/L+ HSA 0.5g/L	212.4	155.5	108.5	143%	1 : 5
LF 0.5g/L+ HSA 0.5g/L	314.9	258.0	158.9	163%	1: 1
LF 0.8g/L+ HSA 0.5g/L	302 8	245.9	167.4	147%	1: 0 625
LF 1.5g/L+ HSA 0.5g/L	336 8	279.9	181.7	154%	1: 0 33

LF 0.1g/L+ HSA 1.0g/L	239 7	182.8	135.7	133	1: 10
LF 0.5g/L+ HSA 1.0g/L	339 4	282.5	186.1	152	1: 2
LF 0.8g/L+ HSA 1 0g/L	357.7	300.8	194.6	155	1: 1.2
LF 1 5g/L+ HSA 1 0g/L	343.7	286.8	208.9	137	1: 0.66
LF 0.1g/L+ HSA 2.0g/L	261.3	204.4	195.4	105	1: 20
LF 0.5g/L+ HSA 2.0g/L	377 2	320.3	245.8	131	1: 4
LF 0.8g/L+ HSA 2.0g/L	414 8	357.9	254.3	141	1: 2.4
LF 1.5g/L+ HSA 2.0g/L	358.0	301.1	268.6	112	1: 1.33

Discussion: Ratios of lactoferrin to albumin as low as 1 to 0.333 to as high as 1: 20 produced synergistic stimulations of cell growth. Optimal ratios of lactoferrin to albumin were found at 1 to 0.333 wt/wt (at an albumin concentration of 0.5 g/L), at 1 to 1.2 wt/wt (at an albumin concentration of 1.0 g/L); and at 1: 2.4 wt/wt (at an albumin concentration of 2.0 g/L).

Thus the stimulation of cell productivity occurs over a broad range of ratios of recombinant albumin and lactoferrin. Optimal ratios of lactoferrin and recombinant albumin vary to a certain extend on the absolute concentration of both protein components. Preferred ratios of Lactoferrin to Albumin range from about 1 (lactoferrin) to 0.33(albumin) to about 1 (lactoferrin) to 3 (albumin) wt/wt.

Example 13: Evaluation of the effect of combinations of albumin and transferrin in the presence of other cell culture components.

To determine the effect of other cell culture components on the synergistic effect of lactoferrin and albumin on cell growth and viability, a series of additional experiments were carried out with other cell culture components which have been previously shown to be essential for cell growth in serum free conditions.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments transferrin (TF) was used at an initial concentration of 5.5 µg/mL; Plant derived recombinant

Human Serum Albumin (HSA) was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite (SE) and Ethanolamine were used at concentrations of 1:500,000 v/v and 6 7 µg/mL respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the supplements listed above, as described in **Table E12** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C. 6% CO_2, in a humidified environment.

TABLE E12

	Viable cells/ ml	Std	Observed Effect	Predicted Effect	Synergistic	Ratio of transferrin to albumin
DMEM/F12 alone	0.01	0.01				
10% FBS	11.40	0 71	11.39			
TF 5.5 µg/L	0.02	0.00	0.01			
SE 6.7 µg/L	0.22	0.13	0.21			
HSA 0.1 g/L	2.31	0.06	2.30			
HSA 0.5 g/L	4.67	0.18	4.66			
HSA 1 g/L	4.55	0.46	4.54			
doubles						
TF+SE	0.40	0.04	0.38	0.21	yes	
TF+ HSA 0.1 g/L	3.00	0.02	2.98	2.30	yes	1:18
TF+ HSA 0.5 g/l	5.42	0.20	5.41	4.67	yes	1:90
TF+ HSA 1 g/L	5.71	0.14	5.69	4.55	yes	1:181
doubles						
SE+ HSA 0.1 g/l	2.76	0 06	2.75	2.50	Yes/no	
SE+ HSA 0.5 g/L	4.15	0.16	4.14	4.87	no	

SE+ HSA 1 g/L	3.75	0.22	3.74	4.75	no	
Triples						
TF+SE+ HSA 0.1 g/L	7.40	0.00	7.39	2 51	yes	*1:18*
TF+SE+ HSA 0.5 g/L	9.48	0.31	9.46	4.87	yes	*1:90*
TF+SE+ HSA 1 g/L	9.71	0.11	9.70	4.75	yes	*1 181*

Discussion: Recombinant human serum albumin in combination with recombinant transferrin showed synergistic effects when added to culture media together. This effect was further augmented in the presence of selenium. However the addition of selenium did not act synergistically with recombinant plant derived human serum albumin alone. Maximal synergistic effects of transferrin and plant derived recombinant human serum albumin occurred in the presence of selenium and at ratios of transferrin to plant derived recombinant human serum albumin of about 1 (transferrin) to 100 (albumin) wt/wt. and of about 1 (transferrin) to 200 (albumin).

Selenium is a well known essential trace element for cell growth and development, and its positive role in biological systems includes detoxification of free radicals by activating glutathione peroxidase. Additionally selenium can act as a highly effective iron carrier in serum free media, which helps to explain its synergistic impact on transferrin stimulated cell growth. (Zhang et al., Biotechnol. Bioeng. (2006) 95(6) 1188-97)

Example 14: Evaluation of the effect of combinations of albumin, transferrin and insulin.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin, plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination with insulin was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 0.1 g/L, 0.5 g/L, and 1 g/L & Sodium Selenite and Ethanolamine were used at concentrations of 6.7 µg/mL and 1:500,000 v/v and respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E13** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment, and shaken with 2 cm rotational orbit at 120 RPM.

TABLE E13

	Viable cells/ml	Std	Observed Effect	Predicted Effect	Synergistic
D/F12 alone	0.01	0.01			
10% FBS	11.65	0.21	11.64		
Factors Alone					
Insulin (IN) 10 µg/L	0.04	0.02	0.03		
Transferrin (TF) 5.5 µg/L	0.02	0.00	0.01		
Sodium Selenite (SE) 6.7 µg/L	0.22	0.13	0.21		
HSA 0.1 g/L	2.31	0.06	2.30		
HSA 0.5 g/L	4.67	0.18	4.66		
HSA 1 g/L	4.55	0.46	4.54		
Doubles					
IN+TF	0.03	0.02	0.02	0.06	
IN+SE	1.06	0.15	1.05	0.26	Yes
IN+ HSA 0.1 g/L	2.54	0.08	2.53	2.35	No
IN+ HSA 0.5 g/L	3.96	0.34	3.95	4.71	No
IN+ HSA 1 g/L	4.61	0.16	4.60	4.59	No
Triples					
IN+TF+SE	2.13	0.49	2.12	0.28	Yes
IN+TF+ HSA 0.1 g/L	3.29	0.03	3.28	2.37	Yes
IN+TF+ HSA 0.5 g/L	5.84	0.49	5.83	4.73	Yes
IN+TF+ HSA 1 g/L	6.84	0.34	6.83	4.61	Yes

Triples					
IN+SE+ HSA 0.1 g/L	2.21	0.17	2.19	2.37	No
IN+SE+ HSA 0 5 g/L	3.41	0.21	3.39	4.73	No
IN+SE+ HSA 1 g/L	3 62	0.23	3.60	4.61	No
Quaternaries					
IN+TF+SE+ HSA 0 1 g/L	7.91	0.46	7 90	2 59	Yes
IN+TF+SE+ HSA 0 5 g/L	9.14	0.63	9 13	4 95	Yes
IN+TF+SE+ HSA 1 g/L	10.07	0.67	10.05	4.83	Yes

Discussion: In these studies the addition of insulin and plant derived recombinant human serum albumin did not produce a synergistic effect on cell growth. By contrast the addition of plant derived recombinant human serum albumin and transferrin and insulin did produce a synergistic effect on cell growth.

Example 15: Evaluation of the effect of combinations of albumin, transferrin and insulin in the presence of Selenium.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin,(HSA) insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 6 7 µg/m L Sodium Selenite at 0.4x 10^5 viable cells/mL In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL Ethanolamine were used at a concentration of 1·500,000 v/v The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E14** below. Control cultures without added supplements were used as the baseline All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E14						
Condition	Viable cell count 10e5	Std	Observed Effect	Predicted Effect	% Improvement over predicted activity	Synergistic
Base medium of D/F12+ Selenium	0.08	0.04	0.00			
10% FBS	13.20	0 28	13.12			
Insulin	0.30	0.09	0.22			
Factors Alone						
TF	0.27	0.04	0.19			
HSA 0.1 g/L	2.23	0.11	2.15			
HSA 0.5 g/L	6.21	0.20	6 13			
HSA 1 g/L	8.00	0 05	7 91			
doubles						
IN+TF	0.48	0.02	0.40	0.41		No
IN+ HSA 0.1 g/L	1.81	0.02	1.72	2.37		No
IN+ HSA 0.5 g/L	6.00	0.09	5.91	6.35		No
IN+ HSA 1 g/L	7.43	0 19	7.35	8.13		No
doubles						
TF+ HSA 0 1 g/L	4.29	0.04	4.20	2.34	179	Yes
TF+ HSA 0 5 g/L	12.35	0.21	12.27	6.32	194	Yes
TF+ HSA 1 g/L	15.48	0.71	15.39	8.1	190	Yes
Triples						
IN+TF+ HSA 0.1 g/L	4.18	0.21	4.09	2.56	159	Yes
IN+TF+ HSA 0.5 g/L	13.60	0.00	13.52	6.54	206	Yes
IN+TF+ HSA 1 g/L	15.38	0 22	15.29	8.32	184	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin.

Example 16: Evaluation of the effect of combinations of albumin and transferrin in the presence of Ethanolamine.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin (HSA), insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 1:500,000 Ethanolamine v/v at 0.4x 10^5 viable cells/ml. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite was used at a concentration of 6.7 µg/mL. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E15** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E15				
Condition	Observed Effect (Viable cells/ml)	Std	Predicted Effect (Viable cells/ml)	Synergistic
Base Medium of D/F12+ Ethanolamine	0.51	0.16		
10% FBS	14.45	0.64		
IN	1.41	0.24		
Factors Alone				
TF	2.08	0.15		
HSA 0.1 g/L	2.16	0.07		
HSA 0.5 g/L	5.66	0.35		
HSA 1 g/L	7.08	0.21		
doubles				
IN+TF	3.12	0.11	3.48	No
IN+ HSA 0.1 g/L	1.87	0.00	3.57	No
IN+ HSA 0.5 g/L	4.48	0.25	7.07	No
IN+ HSA 1 g/L	5.14	0.19	8.49	No
doubles				
TF+ HSA 0.1 g/L	6.72	0.35	4.24	Yes
TF+ HSA 0.5 g/L	12.30	0.26	7.74	Yes
TF+ HSA 1 g/L	16.20	0.65	9.16	Yes
Triples				
IN+TF+ HSA 0.1 g/L	7.46	0.28	5.64	Yes
IN+TF+ HSA 0.5 g/L	13.30	0.36	9.15	Yes
IN+TF+ HSA 1 g/L	16.60	0.48	10.57	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin, over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin under these conditions.

Surprisingly the combination of Insulin, transferrin and plant derived recombinant human serum albumin produced superior cell growth compared to 10 % serum, demonstrating the superior properties of these combinations of proteins.

Example 17: Comparison of rice recombinantly produced albumin and plasma derived albumin.

To directly compare the effect of rice derived recombinant human serum albumin (rHSA) to plasma derived serum albumin (pHSA), samples of both were directly compared at two ratios when mixed with rice derived recombinant transferrin for their ability to stimulate cell growth.

Methods: Hybridoma cells were grown in DMEM/F12 (Gibco) in 10% FBS. Cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in either 1)DMEM/F12 base medium, 2) DMEM/F12+sodium selenate base medium or 3) DMEM/F12 base medium at $0.75x10^5$ viable cells/ml. Each base medium was further supplemented with either recombinant transferrin (TF), rice derived recombinant human serum albumin (rHSA), plasma derived human serum albumin (pHSA) or combinations of both transferrin and either form of albumin. After 3 days of incubation at 37° C, 5% CO_2, the concentration of viable cells was determined by a Guava PCA cell counter as instructed by the manufacturer. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components. The Effect Factor was calculated as the observed effect divided by the expected effect. Effect factor of 1 is indicative of expected effect (additive). Effect factor >1 is indicative of more than additive or synergistic effect.

In these experiments transferrin (TF) was used at an initial concentrations of 1 and 3 mg/L; albumins were used at concentrations of 5 g/L, Sodium Selenite was used at a concentration of

6.7 µg/L & Ethanolamine was used at 1:500,000 v/v.

Table E16					
Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual / expected
Base Medium	0.11	0.07			
Components alone					
rTF 1 mg/L	0.21	0.01	0.10		
rTF 3 mg/L	0.28	0.09	0.18		
pHSA 5 g/L (Seracare)	11.30	0.28	11.19		
rHSA 5 g/L (Cellastim)	5.11	0.02	5.00		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	12.85	0.49	12.74	11.51	1.1
rTF3 mg/L + pHSA 5 g/L	13.20	0.42	13.09	16.41	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	18.80	0.71	18.69	5.31	3.5
rTF3 mg/L + rHSA 5 g/L	18.85	0.92	18.74	5.39	3.5

Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual/expected
Base Medium	0.04	0.02			
Components alone					
rTF 1 mg/L	0.14	0.01	0.10		
rTF 3 mg/L	0.14	0.05	0.10		
pHSA 5 g/L	6.08	0.13	6.04		
rHSA 5 g/L	1.76	0.17	1.72		

pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	7.46	0.49	7.42	6.22	1.2
rTF3 mg/L + pHSA 5 g/L	7.84	0.05	7.79	7.84	1.0
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	8.55	0.79	8.51	1.90	4.5
rTF3 mg/L + rHSA 5 g/L	8.37	0.23	8.33	1.90	4.4

Base Medium:DMEM/F12	**Viable cells/ml 10e5**	**Std**	**Observed Effect**	**Predicted Effect**	**Effect Factor (actual/ expected**
Base Medium	1.01	0.02			
Components alone					
rTF 1 mg/L	3.45	0.27	2.44		
rTF 3 mg/L	3.47	0.27	2.46		
pHSA 5 g/L	13.15	0.49	12.14		
rHSA 5 g/L	3.46	0.13	2.45		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	14.83	0.49	13.82	16.60	0.8
rTF3 mg/L + pHSA 5 g/L	14.57	0.12	13.56	16.61	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	17.87	1.46	16.86	6.91	2.4
rTF3 mg/L + rHSA 5 g/L	20.20	1.13	19.19	6.93	2.8

Discussion: Table E14 shows the results of three independent experiments that confirm that the combinations of TF with rice derived recombinant human serum albumin produced Observed Effect values much greater than the Predicted Effect. By comparison, the combination of ***plasma***-derived HSA and transferrin produced values that were similar to, or less than, the expected effect. Thus the ability of albumin to synergistically enhance the activity of transferrin

and lactoferrin appears to be dependent on the source of albumin, rather than being an inherent characteristic of the albumin itself Specifically the present experiments establish that the synergistic properties of human serum albumin with transferrin related proteins such as transferrin and lactoferrin is an unexpected and surprising property of rice derived recombinant human serum albumin. Significantly the combination of transferrin and plant derived recombinant albumin was more effective in stimulating cell growth than the combination of plasma derived albumin and transferrin, even though plasma derived albumin was more effective at 5 g/L alone than the same concentration of rice derived human serum albumin.

Example 18: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with either plasma derived or recombinant transferrin.

To directly compare the effect of rice derived recombinant human transferrin (rTF) to plasma derived transferrin (pTF), samples of both were directly compared when mixed with rice derived recombinant human serum albumin for their ability to stimulate cell growth.

Methods: A base medium (BM) was composed that consisted of DMEM/F12+ with Sodium Selenite 0.0067 mg/L, Ethanolamine 1:500,000 v/v. Base medium included human plasma transferrin, pTF at a concentration of 5.5 mg/L in combination with either plasma HSA (pHSA, cell culture grade Seracare) or rice derived recombinant HSA (rHSA). Hybridoma cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in at $0.80x10^5$ viable cells/ml and incubated at 37° C, 6% CO_2, The concentration of viable cells was determined daily by a Guava PCA cell counter as instructed by the manufacturer. The concentration of antibody produced by the cells and secreted into the medium was determined on day 6 by quantitative ELISA (Bethyl laboratories) Error bars indicate standard deviation The production of antibody with the combination of pTF +rHSA was similar to that of DMEM/F12+10% FBS.

Discussion: ~~**Figure**~~**FIG. 13** shows that rice derived recombinant human serum albumin was able to act synergistically with plasma derived transferrin to significantly enhance the growth and productivity of the cells. By comparison, plasma derived serum albumin was unable to exert a similar stimulatory effect. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective with transferrin related proteins that were either plasma derived, or recombinant.

Example 19: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with insulin related growth factor-1.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components such as the growth factor IGF-1, additional experiments were performed to assess this possibility.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) as described previously. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and recombinant insulin related growth factor 1 (IGF1) (Ajinomoto) alone or in combination were examined Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1 5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or IGF-1 or various mixtures of the two additives. Control cultures without added rHSA or IGF-1 were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture All cultures were grown at 37°C, 6% CO_2, in a humidified environment. Table E17 shows the results of the experiment. Here the Observed Effect was calculated as the net viable cell concentration (observed-base medium), and the Predictive Effect was calculated as the sum of the effect of the individual components.

Table E17

	viable cells/ml 10e5	Observed Effect (Cell count-baseline)	Predicted Effect
No Additive(baseline)	3 3	0.0	
HSA 0.5 g/L	7.3	4 0	
HSA 1g/L	7.9	4.6	
IGF-1 30 nM	12.0	8.7	

HSA 0.5 g/L+ IGF-1 30nM	23.5	15.6	12.7
HSA 1 g/L+ IGF-1 30nM	25.6	13.6	13.3

Results and Discussion: **Table E17** shows that rice derived recombinant human serum albumin was able to act synergistically with recombinant IGF-1 to significantly enhance the growth and productivity of the cells. By comparison, recombinant albumin did not act synergistically with insulin (Table E15) suggesting that synergism is not a general effect across all cell culture components. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective in combination with IGF-1.

It will, of course, be appreciated that the above description has been given by way of example only and that modifications in detail may be made within the scope of the present invention.

The invention is capable of considerable modification, alteration, and equivalents in form and function, as will occur to those ordinarily skilled in the pertinent arts having the benefit of this disclosure.

While the present invention has been described for what are presently considered the preferred embodiments, the invention is not so limited. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the detailed description provided above.

ABSTRACT OF THE DISCLOSURE

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

Electronic Acknowledgement Receipt	
EFS ID:	33124416
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition for review by the Office of Petitions	PTO_Renewed_Petition_09Jul18_50091-CNT_1128818.PDF	92046 [illegible]	no	2

Warnings:

Information:					
2		PTO_Amendment_and_Statement_09Jul18_50091-CNT_1128816.PDF	79430	yes	3
	Multipart Description/PDF files in .zip description				
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	Preliminary Amendment		1	1	
	Specification		2	2	
	Applicant Arguments/Remarks Made in an Amendment		3	3	
Warnings:					
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3	Specification	PTO_Substitute_Specification_Clean_Copy_09Jul18_50091-CNT_1128812.PDF	919115	no	126
Warnings:					
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Application No. 15188478

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

[X] **GRANTED**

[ ] **DISMISSED**

[ ] **DENIED**

Office of Petitions: Decision Count Sheet

Mailing Month: 7

Application No.: 15188478

15188478

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: Sherry D. Brinkley

Count (1) - Palm Credit 15188478

Decision: GRANT

FINANCE WORK NEEDED
☐ Select Check Box for YES

GRANT

Decision Type: 502 - 37 CFR 1.137(b) - REVIVAL BASED ON UNINTENTI

502

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

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☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Petti	50091-CNT1	8304

23589 7590 07/13/2018

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
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EXAMINER	
BOLLAND, PAUL I	

ART UNIT	PAPER NUMBER
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Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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In re Application of	:	
Steven Clyde Petitit, et al.	:	
Application No. 15/188,478	:	ON PETITION
Filed: June 21, 2016	:	
Attorney Docket No.: 50091-CNT1	:	

This is a decision on the renewed petition to revive the above-identified application, filed July 9, 2018, under the unintentional provisions of 37 CFR 1.137(a).

The petition is **GRANTED**.

The application became abandoned for failure to reply in a timely manner to the Notice to File Missing Parts of Nonprovisional Application (Notice), mailed July 1, 2016. A Notice of Abandonment was mailed on March 2, 2017. On March 16, 2017, a petition under 37 CFR 1.137(a) was filed; however, the petition was dismissed in a corrected decision mailed May 10, 2018. On July 9, 2018, the present petition was filed.

On reconsideration, the petition is found to comply with all the requirements of 37 CFR 1.137(a)

The application is being referred to the Office of Patent Application Processing (OPAP) for further pre-examination processing.

Telephone inquiries related to this decision should be directed to the undersigned at (571) 272-3204. Telephone inquiries related to processing at OPAP should be directed to their hotline at (571) 272-4000.

/SDB/
Sherry D. Brinkley
Lead Paralegal Specialist
Office of Petitions

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET NO	TOT CLAIMS	IND CLAIMS
15/188,478	06/21/2016	1656	1000	50091-CNT1	10	1

CONFIRMATION NO. 8304

UPDATED FILING RECEIPT

23589
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

Date Mailed: 07/18/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Steven Clyde Pettit, Fort Collins, CO;
Mary Ann Michelle Fernandez Santos, Elk Grove, CA;
Ning Huang, Davis, CA;

Applicant(s)

Ventria BioScience Inc., Fort Collins, CO

Power of Attorney: The patent practitioners associated with Customer Number 23589

Domestic Priority data as claimed by applicant

This application is a CON of 12/708,462 02/18/2010 ABN
which claims benefit of 61/154,204 02/20/2009

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 06/30/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/188,478**

Projected Publication Date: None, application abandoned.

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies. particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired betore the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation. and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/188,478

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b) or (c))	N/A	N/A	N/A	75		N/A	
SEARCH FEE (37 CFR 1.16(k), (l) or (m))	N/A	N/A	N/A	330		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p) or (q))	N/A	N/A	N/A	380		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	10 minus 20 =	*	x 50 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x 230 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C 41(a)(1)(G) and 37 CFR 1.16(s).			200			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL	985		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	*	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNTI

CONFIRMATION NO. 8304

23589
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

INFORMAL NOTICE

OC000000101013728

Date Mailed: 07/18/2018

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

A new inventor's oath or declaration that identifies this application (e.g., by Application Number and filing date) is required. The inventor's oath or declaration does not comply with 37 CFR 1.63 in that it:

- does not state that the above-identified application was made or authorized to be made by the person executing the oath or declaration.

  Steven Clyde Pettit
  Mary Ann Michelle Fernandez Santos
  Ning Huang

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/kgebremichael/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478		Steven Clyde Pettit	50091-CNT1

CONFIRMATION NO. 8304

23589
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

WITHDRAWAL NOTICE

OC000000101004898

Date Mailed: 07/18/2018

Letter Regarding a New Notice and/or the Status of the Application

If a new notice or Filing Receipt is enclosed, applicant may disregard the previous notice mailed on 03/02/2017. The time period for reply runs from the mail date of the new notice. Within the time period for reply, applicant is required to file a reply in compliance with the requirements set forth in the new notice to avoid abandonment of the application.

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web. https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If the reply is not filed electronically via EFS-Web, the reply must be accompanied by a copy of the new notice.

If the Office previously granted a petition to withdraw the holding of abandonment or a petition to revive under 37 CFR 1.137, the status of the application has been returned to pending status.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**

/kgebremichael/

Electronic Acknowledgement Receipt

EFS ID:	33467741
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	21 AUG-2018
Filing Date:	21-JUN-2016
Time Stamp:	15:23:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	Executed_Declarations_Pettit_Santos_Huang_50091-CNT.PDF	3658094	no	3

Warnings:

Information:					
2	Miscellaneous Incoming Letter	LTPTO_Submission_of_Missing_Requirements_21AUG2018_50091-CNT.PDF	86521	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3744615		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/188,478 filed on June 21, 2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Steven Clyde Pettit Date (Optional): August 2, 2018

Signature: Steven Clyde Pettit

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/01 (06-12)
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This declaration is directed to:
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- ☒ United States application or PCT international application number 15/188,478 filed on June 21, 2016.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

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LEGAL NAME OF INVENTOR

Inventor: Mary Ann Michelle Fernandez Santos Date (Optional): 03/03/18

Signature: Mary Ann Michelle Fernandez Santos

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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LEGAL NAME OF INVENTOR

Inventor: Ning Huang Date (Optional): Aug 10, 2016

Signature:

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Art Unit: 1656
Filing Date: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. Holland

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

TRANSMITTAL OF INVENTOR DECLARATIONS

In response to the "Informational Notice to Applicant" dated July 18, 2018 (copy appended), please find enclosed the requisite Inventor Declarations, now in compliance with 37 CFR 1.63.

Applicant notes that the surcharge for late submission of these documents, as required by 37 C.F.R. § 1.492(e), was paid with the initial filing of this application

Should any further fees be required, the Office is authorized to deduct the same from Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Cook/

By _____

Crissa A. Cook, Reg. No. 64,054
HOVEY WILLIAMS LLP
10801 Mastin Boulevard, Suite 1000
Overland Park, KS 66210
913-647-9050
ATTORNEYS FOR APPLICANT(S)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
[illegible]
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNTI

CONFIRMATION NO. 8304

23589
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

INFORMAL NOTICE

OC000000101013728

Date Mailed: 07/18/2018

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

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- does not state that the above-identified application was made or authorized to be made by the person executing the oath or declaration.

  Steven Clyde Pettit
  Mary Ann Michelle Fernandez Santos
  Ning Huang

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/kgebremichael/

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APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

23599 7590 10/16/2018

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER
ROLLAND, PAUL F

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
10/16/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

uspatents@hoveywilliams.com

Office Action Summary	Application No. 15/188,478	Applicant(s) Pettit et al.	
	Examiner PAUL J HOLLAND	Art Unit 1656	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 03/16/2017.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b)☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 35-44 is/are pending in the application.
5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 35-44 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
a)☐ All b)☐ Some** c)☐ None of the:
1.☐ Certified copies of the priority documents have been received.
2.☐ Certified copies of the priority documents have been received in Application No. _____.
3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)
2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____
3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____
4) ☐ Other: _____

DETAILED CORRESPONDENCE

Application Status

1. The present application is being examined under the pre-AIA first to invent provisions.

2. Claims 35-44 are pending.

Priority

3. Acknowledgement is made of this continuation of U.S. Non-provisional Application No. 12/708,462, filed on 02/18/2010, which claims domestic priority to U.S. Provisional Application No. 61/154,204, filed on 02/20/2009.

Specification/Drawings

4. The specification and drawings are objected to under 37 CFR 1.821(a)(1) and (a)(2). This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 CFR 1.821(a)(1) and (a)(2). However, this application fails to comply with the requirements of 37 CFR 1.821 through 1.825; applicants' attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990). To be in compliance, applicants should identify nucleotide sequences of at least 10 nucleotides and amino acid sequences of at least 4 amino acids in the specification by a proper sequence identifier, i.e., "SEQ ID NO:" (see MPEP 2422.01). If these sequences have not been listed in the computer readable form and paper copy of the sequence listing, applicant must provide an initial computer readable form (CRF) copy of the "Sequence Listing", an initial paper copy of the "Sequence Listing", as well as an amendment directing its entry into the specification, and a statement that the content of the paper and CRF copies are the same and, where applicable, include no new matter as required

by 37 C.F.R. 1.821(e) or 1.821(f) or 1.821(g) or 1.821(b) or 1.825(d). In the instant case, Figures 1 and 5 of the specification and drawings contain sequences that are encompassed by the definition of amino acids and nucleotides without appropriate sequence identifiers.

5. The specification is further objected to because the status of the parent 12/708,462 in the CROSS REFERENCE TO RELATED APPLICATIONS section of the specification does not reflect the abandonment of this application.

Claim Rejections - 35 USC § 112(b), or Second Paragraph

6. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.--The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

7. Claims 35-44 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

Regarding claim 35 (claims 36-44 dependent therefrom), the term "improves" is a relative term which renders the claim indefinite. The term "improves" is a term of degree and the examiner has reviewed the specification and can find no examples or teachings that can be used for ascertaining the variance intended by the recited term of degree. Moreover, there is nothing in the specification or prior art of record to indicate that one of ordinary skill in the art could have ascertain the scope of the recited degree and what the improvement is being

compared to. It is suggested that applicant clarify the meaning of the claims. See Supplementary Examination Guidelines for Determining Compliance with 35 U.S.C. §112 and for Treatment of Related Issues in Patent Applications, 76 FR 7162 (Feb. 9, 2011), page 7165.

Furthermore regarding claims 43-44, a broad range or limitation together with a narrow range or limitation that falls within the broad range or limitation (in the same claim) is considered indefinite, since the resulting claim does not clearly set forth the metes and bounds of the patent protection desired. See MPEP § 2173.05(c). Note the explanation given by the Board of Patent Appeals and Interferences in *Ex parte Wu*, 10 USPQ2d 2031, 2033 (Bd. Pat. App. & Inter. 1989), as to where broad language is followed by "such as" and then narrow language. The Board stated that this can render a claim indefinite by raising a question or doubt as to whether the feature introduced by such language is (a) merely exemplary of the remainder of the claim, and therefore not required, or (b) a required feature of the claims. Note also, for example, the decisions of *Ex parte Steigewald*, 131 USPQ 74 (Bd. App. 1961); *Ex parte Hall*, 83 USPQ 38 (Bd. App. 1948); and *Ex parte Hasche*, 86 USPQ 481 (Bd. App. 1949). In the present instance, claim 43 recites the broad recitation 1 to 5000, and the claim also recites 1 to 50 which is the narrower statement of the range/limitation. Claim 44 recites the broad recitation 1 to 3, and the claim also recites 1 to 0.33, which is the narrower statement of the range/limitation. It is suggested that applicants clarify the meaning of the claims.

Claim Rejections - 35 USC § 101

8. 35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

9. Claims 35-44 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. Claim(s) 35-44 are directed to a supplement or complete medium comprising a recombinant albumin wherein said recombinant albumin is: i) produced in a plant; ii) has less than 1 EU of endotoxin/mg of albumin; and iii) less than 2% aggregated albumin; and wherein said supplement or complete medium improves growth of a cell in cell culture medium comprising said supplement or complete medium. The claim(s) does/do not include additional elements that are sufficient to amount to significantly more than the judicial exception because claims encompass an albumin protein that is not markedly different in structure from that which is found in nature, such as rice albumin produced by *Oryza sativa* as disclosed by Mawal et al. (Bioscience Reports, 1987; examiner cited) or human serum albumin. Indeed, the specification defines "albumin" as encompassing naturally occurring albumin [see p. 48 of the disclosure]. The recitation of "recombinant" or "produced in plants" is not sufficient to transform the supplement into something that is markedly different in structure because plants naturally undergo recombination, and furthermore, the albumin even if it is recombinantly produced in a heterologous host cell, would still be identical in structure to albumin that is found in nature. The dependent claims do not add anything significantly more to the naturally occurring albumin because claims 36-40 recite naturally occurring heat shock proteins that exist in rice. Also, dependent claims 41-44 recite transferrin and lactoferrin, which are not markedly different in structure than transferrin and lactoferrin that exist in human blood and would come in contact with human serum albumin. It is suggested that

applicants amend the claims to demonstrate that the claimed proteins are markedly different in structure from those proteins which are naturally occurring.

Claim Rejections - 35 USC § 102

10. The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

11. Claims 35-40 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Mawal et al. (*Bioscience Reports*, 1987; examiner cited) as evidenced by Davies (**US Patent Application** 5,917,022; examiner cited).

12. Claims 35-40 are drawn to a supplement or complete medium comprising: a recombinant albumin wherein said recombinant albumin is: i. produced in a plant; ii. has less than 1 EU of endotoxin/mg of albumin; and iii. less than 2% aggregated albumin; and wherein said supplement or complete medium improves growth of a cell in a cell culture medium comprising said supplement or complete medium.

13. With respect to claims 35-40, Mawal et al. teach a rice albumin from *Oryza sativa* that accounts for about 5% of the total seed proteins [see Abstract]. The rice albumin taught by Mawal et al. is interpreted as a supplement comprising recombinant albumin because plants undergo recombination as a natural process and a supplement can broadly and reasonably interpreted as any compound that can be added to anything. Indeed the entire rice seed or plant can be interpreted as a supplement. Although Mawal et al. does not explicitly teach that

the supplement has less than about 1 EU endotoxin/mg of albumin, evidentiary reference Davies is cited in accordance with MPEP 2131.01.II to demonstrate that endotoxin is only found as lipopolysaccharides in the cell wall of Gram-negative bacteria [see Column 1, lines 17-48]. Since rice albumin comes from a rice plant and is not a bacteria, then the albumin taught by Mawal et al. would inherently lack any endotoxin. Although Mawal et al. does not explicitly teach that the supplement is less than about 2% aggregated albumin, and wherein said supplement or complete medium improves growth of a cell in cell culture medium comprising said supplement or complete medium; wherein said recombinant albumin comprises a heat shock protein selected from the group consisting of Rice HSP70, rice endosperm lumenal binding protein, Rice (gblACJ54890.11), EEC69073/OSI_37928, and AAB63469, it is the examiner's position that absent evidence otherwise that this activity and these proteins would be inherent to the rice seed albumin taught by Mawal et al. because the heat shock proteins are rice heat shock proteins and would therefore be present in *Oryza sativa*. Since the claims do not require that the supplement be actively improving the growth of a cell, and knowing that albumin is a supplement used in cell growth, it is the examiner's position that this would be an inherent feature of the rice seed albumin taught by Mawal et al.

MPEP 2112.I states "[T]he discovery of a previously unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new to the discoverer." *Atlas Powder Co. v. Ireco Inc.,* 190 F.3d 1342, 1347, 51 USPQ2d 1943, 1947 (Fed. Cir. 1999)". Since the Office does not have the facilities for examining and comparing applicants' protein with the protein of the prior art, the burden is on the applicant to show a novel or unobvious difference between the claimed

product and the product of the prior art (i.e., that the rice albumin of the prior art does not possess the same material structural and functional characteristics of the claimed supplement comprising recombinant albumin). See *In re Best*, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and *In re Fitzgerald* et al., 205 USPQ 594.

14. Claims 35-40 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Deeter et al. (**WO**2007/002762 A2; examiner cited) as evidenced by Davies (**US Patent Application** 5,917,022; examiner cited).

15. With respect to claims 35-40, Deeter et al. teach components of cell culture medium produced from plant cells that improve the growth of a cell in the cell culture medium wherein the medium comprises human serum albumin produced in rice [see Abstract; paragraphs 0009 and 0116; Example 7]. Deeter et al. also teach clarifying the extracted rice human serum albumin by filtration and purification to approximately 98% (interpreted as no aggregation) [see paragraph 0116]. Although Deeter et al. does not explicitly teach that the supplement comprises less than about 1 EU of Endotoxin/mg of albumin, the reference of Davies is cited in accordance with MPEP 2131.01.II to demonstrate that endotoxin is only found as lipopolysaccharides in the cell wall of Gram-negative bacteria [see Column 1, lines 17-48]. Since the recombinant human serum albumin is produced in rice and is not a bacteria, then the human serum albumin taught by Deeter et al. would inherently lack any endotoxin.

Regarding claims 36-40, Deeter et al. is silent as to the presence of heat-shock proteins, for example those recited in claims 39 and 40, in the albumin preparation. Deeter's method of preparing recombinant albumin from rice grain [see Example 3 at paragraphs 103-105; Example 7 at 116-117), however, is identical to the one employed by applicants [see Example 3 and

Example 5]. It is the examiner's position that absent evidence otherwise, these proteins would be inherent to the recombinant human serum albumin produced by Deeter et al. MPEP 2112.I states "[T]he discovery of a previously unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new to the discoverer." *Atlas Powder Co. v. Ireco Inc.*, 190 F.3d 1342, 1347, 51 USPQ2d 1943, 1947 (Fed. Cir. 1999)". Since the Office does not have the facilities for examining and comparing applicants' protein with the protein of the prior art, the burden is on the applicant to show a novel or unobvious difference between the claimed product and the product of the prior art (i.e., that the rice albumin of the prior art does not possess the same material structural and functional characteristics of the claimed supplement comprising recombinant albumin). See *In re Best*, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and *In re Fitzgerald* et al., 205 USPQ 594.

16. Claim 35 is rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Berezenko et al. (**US Patent** 6,034,221, 2000; examiner cited).

17. With respect to claim 23, Berezenko et al. teach highly purified albumin that contains no detectable aggregates or endotoxin [see Column 1, lines 40-49; Examples 1 and 2]. Berezenko et al. teaches that highly purified albumin is useful in supplementing media for higher eukaryotic cells [see Column 1, lines 21-23]. Berezenko et al. exemplifies expressing albumin recombinantly [Example 1 at column 5]. Berezenko et al. teach that highly purified albumin "is less likely to cause adverse side effects." [see Column 1, lines 56-57]. Although Berenzenko et al. does not teach that the albumin is produced in a plant, this recitation is a product-by-process claim. MPEP 2113 states "[E]ven though product-by-process claims are limited by and

defined by the process, determination of patentability is based on the product itself. The patentability of a product does not depend on its method of production. If the product in the product-by-process claim is the same as or obvious from a product of the prior art, the claim is unpatentable even though the prior product was made by a different process." *In re Thorpe,* 777 F.2d 695, 698, 227 USPQ 964, 966 (Fed. Cir. 1985)". The Patent Office bears a lesser burden of proof in making out a case of *prima facie* obviousness for product-by-process claims because of their peculiar nature" than when a product is claimed in the conventional fashion. It is suggested that applicants demonstrate that the recited process steps of the product produce something that is markedly different and unexpected from what is obvious over the prior art of record.

Claim Rejections - 35 USC § 103

18. The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

19. Claims 41-44 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Deeter et al. (**WO**2007/002762 A2; examiner cited) as evidenced by Davies (**US Patent Application** 5,917,022; examiner cited) in view of Luyten et al. (**US Patent** 6,617,161, 2003; examiner cited) and Potter et al. (**US Patent** 5,985,289, 1999; examiner cited).

20. The relevant teachings of Deeter et al. as applied to claims 23-28 are set forth above.

With respect to claims 41-44, Deeter et al. teach components of cell culture medium produced from plant cells that improve the growth of a cell in the cell culture medium wherein

the medium comprises the addition of albumin, lactotransferrin, or transferrin recombinantly produced from plants [see Abstract; paragraphs 0006, 0009, 0061, 0071, and 0116; Example 7] at concentrations of 0.01-50 mg/mL of lactoferrin [see Example 5; paragraph 0111].

However, Deeter et al. does not teach the supplement of claim 41 comprising both albumin and lactoferrin; the supplement of claim 42 comprising both albumin and transferrin; the supplement of claim 43, wherein said ratio of said transferrin to said recombinant albumin is 1 to 50, to 1 to 5000; and the supplement of claim 44, wherein said ratio of said lactoferrin to said recombinant albumin is 1 to 3, to 1 to 0.33.

Luyten et al. teach a complete culture medium for mammalian cells comprising 6.25μg (0.00625mg) transferrin and 1.25mg bovine serum albumin per mL, i.e. a ratio of 1 to 200 [see Column 3, lines 16-62; Table 1; see also Example 1, using the medium]. Regarding the full scope of ratios, Luyten et al. teach that the concentrations of the components can be adjusted to suit cell lines as necessary. [Column 3, lines 64-67].

Potter et al. teach that transferrin and lactoferrin are both iron-chelating proteins [see Column 7, lines 15-16]. Lactoferrin is therefore a "transferrin-related protein." Potter et al. also teach that iron-chelating proteins can be employed in culture media at varying concentrations to modulate iron content in the media as necessary for the cells [see Column 6, line 66, through column 7, line 21].

At the time the invention was made it would have been obvious to one of ordinary skill in the art to combine the teachings of Deeter et al., Luyten et al., and Potter et al. according to the teachings of Luyten et al. and Potter et al. because Deeter et al. teach that albumin, transferrin, or lactoferrin produced in plants can be used to improve the growth of cells in a

culture medium. Luyten et al. teach that the albumin and transferrin can be combined in a culture medium for cell growth. Potter et al. teach that lactotransferrin or transferrin can be employed in culture media at varying concentrations to modulate iron content in the media necessary for the cells. An ordinarily skilled artisan would have had a reasonable expectation of success, a reasonable level of predictability, and would have been motivated to combine the teachings of Deeter et al., Luyten et al., and Potter et al. because Luyten et al. acknowledges that albumin and transferrin can be combined in cell culture growth medium and Potter et al. acknowledges that both lactotransferrin and transferrin can be added to culture medium to modulate iron content. Therefore, the above invention would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Furthermore, MPEP 2144.06.I states "It is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of combining them flows logically from their having been individually taught in the prior art." *In re Kerkhoven*, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980) (citations omitted) (Claims to a process of preparing a spray-dried detergent by mixing together two conventional spray-dried detergents were held to be *prima facie* obvious.). See also *In re Crockett*, 279 F.2d 274, 126 USPQ 186 (CCPA 1960) (Claims directed to a method and material for treating cast iron using a mixture comprising calcium carbide and magnesium oxide were held unpatentable over prior art disclosures that the aforementioned components individually promote the formation of a nodular structure in cast iron.); and *Ex parte Quadranti*, 25 USPQ2d 1071 (Bd. Pat. App. & Inter. 1992) (mixture of two known herbicides held *prima facie* obvious)." In the instant case, the

improvement in cell growth by addition of plant derived albumin, lactotransferrin, or transferrin was known in the prior art as taught by Deeter et al. Therefore, it would have been obvious to one of ordinary skill in the art to combine these proteins as part of a culture medium to improve cell growth.

Although the combination of Deeter et al., Luyten et al., and Potter et al. do not explicitly teach the ratios of lactoferrin of 1 to 3, to 1 to 0.33, MPEP 2144.05.II.A states, "Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical. "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller,* 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955)". In the instant case, it would be obvious to one of ordinary skill in the art to determine the appropriate workable ranges based on the teachings of Deeter et al., Luyten et al., and Potter et al.

Conclusion

21. Status of the claims:

Claims 35-44 are pending.

Claims 35-44 are rejected.

No claims are in condition for an allowance.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to PAUL J HOLLAND whose telephone number is (571)270-3537. The examiner can normally be reached on Monday to Friday from 8AM to 5PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

Notice of References Cited	Application/Control No. 15/188,478	Applicant(s)/Patent Under Reexamination	
	Examiner PAUL J HOLLAND	Art Unit 1656	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-5917022-A	06-1999	Davies; Jeffrey Raymond	C07K14/765	210/656
*	B	US-6034221-A	03-2000	Berezenko; Stephen	C07K14/70571	435/69.6
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	WO-2007002762-A2	01-2007	WO	Deeter Scott	C12N5/0018
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Mawal YR et al Biochemical and Immunological Characterization of Rice Albumin. 1987. Bioscience Reports. Vol. 7, No. 1 p. 1-9 (Year: 1987)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST text search (see search notes)	10/11/2018	P.H.
Reviewed search history of 12/708462	10/11/2018	P.H.
Inventor and Assignee search (PALM and EAST)	10/11/2018	P.H.

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	2	"6617161".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:09
S2	2	"6034221".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:10
S3	2	"5986289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:11
S4	2	"5917022".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:14
S5	2	"6733746".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:52
S6	2	"5985289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/21 16:34
S7	11	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S8	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S9	106	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S10	2	S9 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:47

EAST Search History (Prior Art)

S11	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:48
S12	4	S11 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:48
S13	12	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S14	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S15	107	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S16	2	S15 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:39
S17	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S18	4	S17 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:40
S19	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S20	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S21	126	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S22	2	S21 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:23
S23	25	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:24
S24	4	S23 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:24

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(54) Title: COMPONENTS OF CELL CULTURE MEDIA PRODUCED FROM PLANT CELLS

(57) Abstract: The present invention comprises the production of components of cell culture media produced from plant cells and cell culture media containing the same. Heterologous DNA comprising genes encoding the desired component are introduced into plant cells, especially rice, which then produce the desired component. The component can be isolated from the plant cell and combined with other components to form the required cell culture medium.

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Components of Cell Culture Media Produced from Plant Cells

[0001] This application claims priority benefit to U.S. provisional application Serial No. 60/694,236 filed June 28, 2006. The priority application is incorporated herein by reference in its entirety.

1. Field of the Invention:

[0002] The present invention relates to the production and use of recombinant proteins produced by plant cells as components of cell culture media.

2. Background of the Invention:

[0003] Cell culture techniques allow animal or plant cells that are removed from tissues to grow when supplied with the appropriate nutrients and conditions. The cells are capable of dividing and can continue to grow until limited by some culture variables such as nutrient depletion or toxic buildup (Butler, M. & Jenkins, H., Nutritional aspects of growth of animal cells in culture, *J of Biotechnol.* (1989) 12: 97-110). Cell culture techniques have a number of applications including investigation of the normal physiology or biochemistry of cells (Balaban, B. & Urman, B., Embryo culture as a diagnostic tool, *Reprod. Biomed. Online* (2003) 7(6): 671-82), testing the effect of various chemical compounds or drugs on specific cell types (Farkas, D. & Tannenbaum, S.R., In vitro methods to study chemically-induced hepatotoxicity: a literature review, *Curr. Drug Metab.* (2005) 6(2): 111-25), studying the sequential or parallel combination of various cell types to generate artificial tissues (Wang et al., Cartilage tissue engineering with silk scaffolds and human articular chondrocytes, *Biomaterials* (2006)), and synthesizing valuable biologics from large scale cell cultures (Zeilinger et al., Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions, *Altern. Lab Anim.* (2002) 30(5): 525-38). Cell culture techniques have also been used for in vitro fertilization (Blake et al., Protein supplementation of human IVF culture media, *J Assist. Reprod. Genet.* (2002) 19(3): 137-43; Bungum et al., Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study, *Reprod. Biomed. Online* (2002) 4(3): 233-6), stem cell research (Conley et al., Derivation, propagation and differentiation of human embryonic stem cells, *Int. J Biochem. Cell Biol.* (2004) 36(4): 555-67), vaccine production (Chuang

et al., Pharmaceutical strategies utilizing recombinant human serum albumin, *Pharm. Res.* (2002) 19(5): 569-77; GlaxoSmithKline, Publisher. HAVRIX® (Hepatitis A Vaccine, Inactivated) - Prescribing Information (2005), available at http://us.gsk.com/products/assets/us_havrix.pdf; Innis et al., Protection against hepatitis A by an inactivated vaccine, *JAMA* (1994) 271(17): 1328-34; Merck, Publisher. PROQUAD® - Measles, Mumps, Rubella, and Varicella (Oka/Merck) Virus Vaccine Live - Prescribing Information (2005), available at http://www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf; Litwin, J., The growth of Vero cells in suspension as cell-aggregates in serum-free media, *Cytotechnology* (1992) 10(2): 169-74), tissue engineering including artificial skin (Atala, A., Future perspectives in reconstructive surgery using tissue engineering, *Urol. Clin. North Am.* (1999) 26(1): 157-65, ix-x; Sher, et al., Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation, *J Biol. Chem.* (2006) 281(8): 5178-87) and organs (Neronov et al., Integrity of endothelium in cryopreserved human cornea, *Cryo Letters* (2005) 26(2): 131-6; Han, et al., Interleukin-1alpha-induced proteolytic activation of metalloproteinase-9 by human skin, *Surgery* (2005) 138(5): 932-9) and gene and cell therapy (Chadd, H.E. & Chamow, S.M., Therapeutic antibody expression technology, *Curr. Opin. Biotechnol.* (2001) 12(2): 188-94).

[0004] The biologics encompass a broad range of cell products and include specific proteins or viruses that require animal cells for propagation. For example, therapeutic proteins such as monoclonal antibodies can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures (Dewar et al., Industrial implementation of in vitro production of monoclonal antibodies, *Ilar J* (2005) 46(3): 307-13). The number of such commercially valuable biologics has increased rapidly over the last decade and has led to the present widespread interest in mammalian cell culture technology (Mizrahi, A., Biologicals produced from animal cells in culture-an overview, *Biotechnol. Adv.* (1988) 6(2): 207-20).

[0005] The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The need for cell culture, especially at large scale, became apparent

with the need for viral vaccines. Major epidemics of polio in the 1940s and 1950s promoted a lot of effort to develop an effective vaccine. In 1949, it was shown that poliovirus could be grown in cultures of human cells, which led to considerable interest in the development of large quantities of the polio vaccine using cell culture (Ligon, B.L., Thomas Huckle Weller MD: Nobel Laureate and research pioneer in poliomyelitis, varicella-zoster virus, cytomegalovirus, rubella, and other infectious diseases, *Semin. Pediatr. Infect. Dis.* (2002) 13(1): 55-63). The polio vaccine, produced from de-activated virus, became one of the first commercial products of cultured animal cells (Furesz, J., Developments in the production and quality control of poliovirus vaccines - Historical perspectives, *Biologicals* (2006)).

[0006] Many components of cell culture media are obtained from animal sources, including lactoferrin, transferrin and serum albumin. These components are extracted from animal sources, purified, and then combined with other inorganic ingredients to constitute cell culture media (Mizrahi, A. & Lazar, A., Media for cultivation of animal cells: an overview, *Cytotechnology* (1988) 1: 199-214). Cell culture components produced in this way have several undesirable features. For one, there is no way to ensure that extraneous animal products are completely excluded from the component, no matter how precise the purification techniques. Animal-derived components can carry the risk of contamination of pathogens of animal and human origin such as BSE, HIV and hepatitis virus (Chamberland et al., Emerging infectious disease issues in blood safety, *Emerg. Infect. Dis.* (2001) 7(3 Suppl): 552-3; Hepatitis C: A Brief Review, *America's Blood Centers* (October 1998) vol. 1, issue 3 (Strong, M. ed.), available at http://www.americasblood.org/index.cfm?fuseaction=display.showPage&pageID=133; Weinberg et al., Legal, financial, and public health consequences of HIV contamination of blood and blood products in the 1980s and 1990s, *Ann. Intern. Med.* (2002) 136(4): 312-9).

[0007] For another, small proteinaceous particles, such as prions, could be present and current scientific evidence suggests that even a very small number could result in disease. In a recent case, a healthy subject died from possible transmission of variant Creutzfeldt-Jakob disease from blood transfusion (Llewelyn et al., Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion, *Lancet* (2004)

363(9407): 417-21). This raises concerns regarding the consistent safety of blood and blood-derived products (Bird, S.M., Recipients of blood or blood products "at vCJD risk," *BMJ* (2004) 328(7432): 118-9). Because of such safety concerns, regulators are encouraging replacing animal components with recombinant proteins in vaccine production and other applications of cell culture techniques (Egan, W.M., Bovine-derived products used in the manufacture and formulation of vaccines: current policies and issues for the future (2004), available at http://www.fda.gov/cber/summaries/pda092004we.pdf; European Medicine Agency (EMA), CPMP position statement on Crutzfeldt-Jakob disease and plasma-derived and urine-derived medicinal products (2003), available at http://www.emea.eu.int/pdfs/human/press/pos/287902en.pdf; World Health Organization (WHO), Variant Creutzfeldt-Jakob disease (2002), available at http://www.who.int/mediacentre/factsheets/fs180/en/). Further, many users may be hesitant to use products from cell culture media that contain animal components for religious or moral reasons (Sarkar, S., Use of animal products in vegetarians and others, *Anaesthesia* (2005) 60(5): 519-20).

[0008] Therefore, there is a need to produce components for incorporation into cell culture media that is not derived from, or produced by, animal sources, yet still retain their functional value for cell cultures.

SUMMARY OF THE INVENTION

[0009] Accordingly, it is an objective of the invention to provide cell culture media or media supplements containing components derived from plant cells, which when used in cell culture, results in the same or improved growth and productivity of cells as compared to the use of media containing animal-derived components.

[0010] One embodiment of the invention comprises a cell culture media with at least one plant-produced heterologous protein as a cell culture media component.

[0011] Another embodiment of the invention comprises a method for producing an enhanced cell culture media, comprising producing at least one heterologous protein in a plant cell and combining the at least one plant-produced heterologous protein with a cell culture media.

[0011] Another embodiment of the invention comprises a cell culture media produced by a process comprising producing at least one heterologous protein in a plant cell and combining at least one plant-produced heterologous protein with a cell culture media.

[0012] Another embodiment of the invention discloses that in a cell culture media having one or more proteinaceous cell culture media components, the improvement comprising at least one plant-produced heterologous protein as a cell culture media component, wherein the heterologous protein is produced by the following process:

a) transforming a plant cell with a chimeric gene comprising:

(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein; and

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein. The embodiment may optionally include purifying the heterologous protein from the harvested seeds.

[0013] Another embodiment of the invention comprises a method for producing a cell culture media having at least one plant-produced heterologous protein as a cell culture media component, the method comprising:

a) transforming a plant cell with a chimeric gene comprising:

(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein;

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein;

c) harvesting the seeds from the plant; and

d) combining the heterologous protein with a cell culture media.

[0014] Another aspect of the invention comprises a method for creating a recombinant plant cell that can produce components that may be incorporated into cell culture media or supplement for cell culture media.

[0015] Another aspect of the invention comprises a method of transforming a plant cell by incorporating a DNA segment that encodes for a component of cell culture media.

[0016] Another aspect of the invention comprises a method of transforming a rice cell by incorporating a DNA segment that encodes for components of cell culture media such as amino acids, growth factors, and other cell culture proteins including lactoferrin and human serum albumin.

[0017] Another aspect of the invention comprises a cell culture medium with components that are derived from plants, and more preferably all the organic ingredients are derived exclusively from non-animal sources.

[0018] Another aspect of the invention comprises a method for the production of cell culture media, the method comprising incorporating into cell culture media components derived from recombinant plant cells.

[0019] Another aspect of the invention comprises of the use of the media supplemented with plant-derived components to improve cell growth and productivity in cell cultures.

[0020] Another aspect of the invention comprises improved cell culture media comprising one or more media ingredients and as a supplement to one or more cell culture media components derived from plant cells.

[0021] Another aspect of the invention comprises a method for achieving high growth rate of culture cells and high productivity of culture cells by culturing the culture cell in the improved cell culture media.

[0022] Another aspect of the invention comprises a method of producing an improved cell culture media comprising a) obtaining a plant cell transformed with a

vector containing a plant-based promoter and a gene encoding a cell culture media component, b) cultivating the transformed plant cells to set seeds, c) harvesting the mature seeds, d) extracting and purifying of the cell culture components from the seed, and e) adding the components to cell culture media.

BRIEF DESCRIPTION OF THE FIGURES

[0023] Figure 1 is a comparison of the codon-optimized epidermal growth factor sequence ("Egfactor") with a native epidermal growth factor sequence ("Native Gene"), aligned to show 53 codons in the mature sequences, with 27 (51%) codon changes and 30 (19%) nucleotide changes.

[0024] Figure 2 is a restriction map of the 3,877 bp plasmid, API303 (pGt1-EGF v2.1), showing an expression cassette for epidermal growth factor (EGF), and containing a rice Gt1 promoter, a Gt1 signal peptide, codon optimized EGF, a Nos terminator and an ampicillin resistance selectable marker.

[0025] Figure 3 is a restriction map of the 4,142 bp plasmid, API270 (pGlb-EGF v2.1), showing an expression cassette for epidermal growth factor ("EGF"), and containing a Glb promoter, a Glb signal peptide, codon optimized EGF, a Nos terminator and an ampicillin resistance selectable marker.

[0026] Figure 4 is a Western blot analysis of recombinant human EGF ("rhEGF") in the R1 generation of transgenic rice seeds. Lane 1 indicates extracts from seeds of control untransformed Taipei 309 rice variety (Oryza sativa, Japonica). Lanes 2 to 5 show rhEGF expressed in the seed extracts obtained from independent transgenic rice events. Lane 6 shows a purified rhEGF standard expressed in yeast, loaded at 125 ng. Lane 7 shows a broad range of molecular weight markers.

[0027] Figure 5 is a comparison of the codon-optimized insulin-like growth factor I sequence ("Insgfact") with a native human insulin-like growth factor I sequence ("native gene"), aligned to show 70 codons in the mature sequences, with 40 (57%) codon changes and 47 (22%) nucleotides changes.

[0028] Figure 6 is a restriction map of the 4,194 bp plasmid, API271 (pGlb-IGF v2.1), showing an expression cassette for insulin-like growth factor I ("IGF"), and containing a Glb promoter, a Glb signal peptide, codon optimized IGF, a Nos terminator and an ampicillin resistance selectable marker.

[0029] Figure 7 is a restriction map of the 3,928 bp plasmid, API304 (pGt1-IFG v2.1), showing an expression cassette for insulin-like growth factor I ("IGF"), and containing a rice Gt1 promoter, a Gt1 signal peptide, codon optimized IGF, a Nos terminator and an ampicillin resistance selectable marker.

[0030] Figure 8 is a Western blot analysis of recombinant human IGF-I ("rhIGF") expressed in the R1 generation of transgenic rice seeds. Lane 1 shows rice seed extract from seeds of control untransformed rice variety Taipei 309. Lanes 2 to 8 show rhIGF expressed in seed extracts obtained from seven independent transgenic rice events. Lane 9 shows a purified rhIGF-1 standard expressed in yeast, loaded at 1 µg. Lane 10 shows a broad range of molecular weight markers.

[0031] Figure 9 is a comparison of the codon-optimized intestinal trefoil factor sequence ("Trefoil") with a native intestinal trefoil factor sequence ("Native Gene"), aligned to show 60 codons in the mature sequences, with 26 (43%) codon changes and 28 (15%) nucleotide changes.

[0032] Figure 10 is a restriction map of the 4,163 bp plasmid, API269 (pGlb-ITF-nos), showing an expression cassette for intestinal trefoil factor ("ITF"), and containing a Glb promoter, a Glb signal peptide, codon optimized ITF, a Nos terminator and an ampicillin resistance selectable marker.

[0033] Figure 11 is a restriction map of the 3,889 bp plasmid, API307 (pGt1-ITF-nos), showing an expression cassette for intestinal trefoil factor (ITF), and containing a rice Gt1 promoter, a Gt1 signal peptide, codon optimized ITF, a Nos terminator and an ampicillin resistance selectable marker.

[0034] Figure 12 is a Western blot analysis of recombinant human ITF ("rhITF") expression in the R1 generation of transgenic rice seeds. Lane 1 indicates extracts from seeds of control untransformed Taipei 309 rice variety. Lanes 2 and 3 show rhITF expressed in the seed extracts obtained from two independent transgenic rice events. Lane 4 indicates a purified rhITF standard expressed in yeast, loaded at 1 µg. Lane 5 shows a broad range of molecular weight markers.

[0035] Figure 13 is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned

to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

[0036] Figure 14 is a plasmid map of the 5,817 bp plasmid, API164 (GT1-Lac), showing an expression cassette for human lactoferrin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human lactoferrin, a Nos terminator and a kanamycin resistance selectable marker.

[0037] Figure 15 shows the results of a SDS-PAGE analysis for the expression of recombinant human lactoferrin. Total proteins from rice seed extracts were suspended in Laemli sample buffer, run on a gradient gel and stained with Coomassie blue. Lane 1 is the molecular weight marker. Lanes 3 to 6 are purified human derived lactoferrin (Sigma Chemical, USA). Lanes 8 to 13 are single seed extracts from homozygous independent transgenic rice lines and lane 14 is a seed extract from non-transformed rice variety Taipei 309.

[0038] Figure 16 is a stable expression of recombinant human lactoferrin in transgenic rice grains from R_2 through R_{10} generations. Total soluble proteins from 1 g of brown rice flour was extracted with 40 ml of extraction buffer and clarified by centrifugation. The extract was analyzed via ELISA. Extraction was repeated three times and standard deviation is shown as an error bar.

[0039] Figure 17 shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human serum albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

[0040] Figure 18 is a plasmid map of the 5,496 bp plasmid, API504 (GT1-HSA), showing an expression cassette for human serum albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human serum albumin, a Nos terminator and a kanamycin resistance selectable marker.

[0041] Figure 19 is a plasmid map of the 12,388 bp plasmid, API508 (JH/GT1-HSA), showing an expression cassette for human serum albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human serum albumin, a Nos terminator and a kanamycin resistance selectable marker.

[0042] Figure 20 shows the effect of three forms of recombinant human lactoferrin on cell growth. The three forms of lactoferrin are asis-lactoferrin, apo-

lactoferrin and holo-lactoferrin. Asis-lactoferrin is the same as partial-lactoferrin with approximately 50% iron saturation. The baseline is with basal media and the rest is baseline supplemented with various concentration of recombinant human lactoferrin as indicated in the graph. Cell growth is measured as thymidine incorporation.

[0043] Figure 21 shows the effect of three forms of recombinant human lactoferrin on cell growth. The three forms of lactoferrin are asis-lactoferrin, apo-lactoferrin and holo-lactoferrin. Asis-lactoferrin is the same as partial-lactoferrin with approximate 50% iron saturation. The baseline is with basal media plus 5% fetal calf serum and the rest are baseline supplemented with epidermal growth factor, 5% FCS or recombinant human lactoferrin at a final concentration of 1 mg/ml. Cell growth is measured as number of cells/ml.

[0044] Figure 22 shows cell culture media supplemented with recombinant human lactoferrin (LACROMIN™) expressed in rice grain promotes hybridoma cell growth and increases hybridoma cell number as compared to animal derived transferrin. FBS is fetal bovine serum. AFM6 is serum-free cell culture media derived from KC Bio, Kansas. AFM6-Fe is a serum-free media without iron.

[0045] Figure 23 shows the effect of recombinant human serum albumin (rHSA) and plasma-derived human serum albumin (pHSA) on the growth of hybridoma AE1 cells in control medium (CM) containing reduced serum (1% FBS).

[0046] Figure 24 shows the effect of recombinant human serum albumin (rHSA) and plasma-derived human serum albumin (pHSA) on the growth of hybridoma AE1 cells in serum-free medium (SMF).

DETAILED DESCRIPTION

[0047] Unless otherwise indicated, all terms used herein have the meanings given below or are generally consistent with same meaning that the terms have to those skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Press, Plainview, N.Y., Ausubel FM et al. (1993) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., and Gelvin and Schilperoot, eds. (1997) Plant Molecular Biology Manual, Kluwer Academic Publishers, The Netherlands for definitions and terms of the art.

[0048] General and specific techniques for producing proteins from plant cells may be obtained from the following patents and applications, each of which is incorporated herein in its entirety by reference: U.S. Pat. Appl. Pub. No. 2003/0172403 A1 ("Plant Transcription Factors and Enhanced Gene Expression"); U.S. Pat. No. 6,991,824 ("Expression of Human Milk Proteins in Transgenic Plants"); U.S. Pat. Appl. Pub. No. 2003/0221223 ("Human Blood Proteins Expressed in Monocot Seeds"); U.S. Pat. Appl. Pub. No. 2004-0078851 ("Production of Human Growth Factors in Monocot Seeds"); U.S. Pat. Appl. Pub. No. 2004/0063617 ("Method of Making an Anti-infective Composition for Treating Oral Infections"); and international application no. PCT/US2004/041083 ("High-level Expression of Fusion Polypeptides in Plant Seeds Utilizing Seed-Storage Proteins as Fusion Carriers"). Other general and specific techniques for producing proteins from plant cells may be obtained, for example, from the following references, each of which is incorporated herein in its entirety by reference: U.S. Patent No. 5,693,507, U.S. Patent No. 5,932,479, U.S. Patent No. 6,642,053, and 6,680,426 (each titled "Genetic Engineering of Plant Chloroplasts"); U.S. Pat. Appl. Pub. No. 2005/0066384 ("Site-Targeted Transformation Using Amplification Vectors"); U.S. Pat. Appl. Pub. No. 2005/0221323 ("Amplification Vectors Based on Trans-Splicing"); U.S. Pat. Appl. Pub. No. 2006/0026718 ("Method of Controlling Cellular Processes in Plants"); and U.S. Pat. Appl. Pub. No. 2006/0075524 (Method of Controlling A Cellular Process In a Multi-Cellular Organism"); Marillonnet et al., Systemic *Agrobacterium tumefaciens*–mediated transfection of viral replicons for efficient transient expression in plants, *Nature Biotech.* (2005) 23(6): 718-723.

[0049] The polynucleotides of the invention may be in the form of RNA or in the form of DNA, and include messenger RNA, synthetic RNA and DNA, cDNA, and genomic DNA. The DNA may be double-stranded or single-stranded, and if single-stranded may be the coding strand or the non-coding (anti-sense, complementary) strand.

[0050] The term "stably transformed" with reference to a plant cell means the plant cell has a non-native (heterologous) nucleic acid sequence integrated into its genome which is maintained through two or more generations.

[0051] By "host cell" is meant a cell containing a vector and supporting the replication and/or transcription and/or expression of the heterologous nucleic acid sequence. Preferably, according to the invention, the host cell is a plant cell. Other host cells may be used as secondary hosts, including bacterial, yeast, insect, amphibian or mammalian cells, to move DNA to a desired plant host cell.

[0052] A "plant cell" refers to any cell derived from a plant, including undifferentiated tissue (e.g., callus) as well as plant seeds, pollen, propagules, embryos, suspension cultures, meristematic regions, leaves, roots, shoots, gametophytes, sporophytes and microspores.

[0053] The term "mature plant" refers to a fully differentiated plant.

[0054] The term "seed product" includes, but is not limited to, seed fractions such as de-hulled whole seed, flour (seed that has been de-hulled by milling and ground into a powder) a seed extract, preferably a protein extract (where the protein fraction of the flour has been separated from the carbohydrate fraction), malt (including malt extract or malt syrup) and/or a purified protein fraction derived from the transgenic grain.

[0055] The term "biological activity" refers to any biological activity typically attributed to that protein by those skilled in the art.

[0056] "Monocot seed components" refers to carbohydrate, protein, and lipid components extractable from monocot seeds, typically mature monocot seeds.

[0057] "Seed maturation" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the

seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

[0058] "Maturation-specific protein promoter" refers to a promoter exhibiting substantially up-regulated activity (greater than 25%) during seed maturation.

[0059] The term "growth factor" refers to proteins, or biologically active fragments thereof, including, without limitation, epidermal growth factor (EGF), keratinocyte growth factors (KGF) including KGF-1 and KGF-2, insulin-like growth factors (IGF) including IGF-I and IGF-II, intestinal trefoil factor (ITF), transforming growth factors (TGF) including TGF-α and -β1-3, granulocyte colony-stimulating factor (GCSF), nerve growth factor (NGF) including NGF-β, and fibroblast growth factor (FGF) including FGF-1-19 and -12β, and biologically active fragments of these proteins. The sequences of these and other growth factors are well-known to those of ordinary skill in the art.

[0060] "Heterologous DNA" refers to DNA which has been introduced into plant cells from another source, or which is from a plant source, including the same plant source, but which is under the control of a promoter that does not normally regulate expression of the heterologous DNA.

[0061] "Heterologous protein" is a protein encoded by a heterologous DNA. The proteins include, but are not limit to, growth factor(s), lactoferrin, transferrin, albumin, insulin, and fractions thereof, growth hormone and fractions thereof, Fibronectin (human) – attachment factor, Lamin (Mouse) – attachment factor, collagenase , platelet derived growth factor, Human brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), thymic factors, haptocorin, lactahedrin, lactoperoxidase. Alpha-fetoprotein, immunoglobin, alpha-lactalbumin.

[0062] As used herein, the terms "native" or "wild-type" relative to a given cell, protein, polypeptide, nucleic acid, trait or phenotype, refers to the form in which that is typically found in nature.

[0063] As used herein, the term "purifying" is used interchangeably with the term "isolating" and generally refers to any separation of a particular component from other components of the environment in which it is found or produced. For example, purifying a recombinant protein from plant cells in which it was produced typically means subjecting transgenic protein-containing plant material to separation techniques such as

sedimentation, centrifugation, filtration, and chromatography. The results of any such purifying or isolating step(s) may still contain other components as long as the results enrich for the component of interest.

[0064] As used herein, the terms "transformed" or "transgenic" with reference to a host cell means the host cell contains a non-native or heterologous or introduced nucleic acid sequence that is absent from the native host cell. Further, "stably transformed" in the context of the present invention means that the introduced nucleic acid sequence is maintained through two or more generations of the host, which is preferably (but not necessarily) due to integration of the introduced sequence into the host genome.

[0065] "Cell culture media" refers to media for cell culture purpose which includes complete media, basal media or basal media supplemented with cell culture media component, cell culture media supplement, or media with various amounts of serum or chemically defined media.

[0066] "Cell culture media component" refers any heterologous proteins for use as a supplement to cell culture media.

[0067] "Cell culture media ingredient" includes cell culture media components, proteins, peptides, hormones, carbohydrates, amino acids, lipids, vitamins, antibiotics, organic and inorganic salts.

[0068] "Cell culture media supplement" refers to a combination of one or multiple cell culture media components with or without other ingredients for addition to cell culture media.

[0069] Most cells in culture grow best at 37°C and at pH 7.4 with appropriate media. Typically, cell culture media contains carbohydrates, amino acids, various salts, bicarbonate to maintain pH, vitamins and hormones, and phenol red as pH indicator. Examples of cell culture media that can be formed include: Dulbecco's Modified Eagle's Medium (DME), Ham's Nutrient Mixtures, MCDB Media, Minimum Essential Medium Eagle, RPMI Media, Ames' Media, BGJb Medium (Fitton-Jackson Modification), Click's Medium, CMRL-1066 Medium, Fischer's Medium, Glascow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), L-15 Medium (Leibovitz),

McCoy's 5A Modified Medium, NCTC Medium, Swim's S-77 Medium, Waymouth Medium, and William's Medium E.

[0070] Frequently, cell culture media is supplemented with serum at concentration of 5 to 10%. Serum is a complex mixture containing undefined essential matter for cell growth. Commonly used serum is fetal calf serum (FCS) and fetal bovine serum (FBS). Due to its nature of complex mixture, lack of consistency and potential risk of pathogen contamination, cell culture industries and regulatory agencies are seeking alternatives to adding serum to cell culture media.

[0071] Since cells grow poorly in basal media, the basal media is then supplemented with various growth factors, hormones, and other essential components to generate serum-free media but maintaining the same cell growth rate as in serum-based media. Human transferrin or bovine transferrin is added as a source of iron. Typically, serum-free media contains insulin, serum albumin, transferrin, insulin-like growth factor and epidermal growth factor. Other proteins such as lactoferrin have also been shown to be able to promote cell growth. Each component provides a distinct function in cell growth. In the art, these protein components are animal-derived. With the increase in the concern of potential pathogen contamination from animal sourced material, cell culture industries are trying to develop animal-component-free media. While some successes have been made with the use of plant-based hydrolysates or recombinant protein produced with microbial system, plant-based hydrolysate is an undefined mixture of components and recombinant protein from microbial sources are too expensive to use in routine cell culture. An alternative source of non-animal-derived components is urgently needed for cell culture industries.

[0072] The cell culture media components produced by the methods to be described in the following sections are entirely plant-based and may be combined with inorganic salts such as NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, and $MgCl_2$ and other ingredients such as amino acids, vitamins, growth factors, sugars, and antibiotics to form a variety of different cell culture media.

[0073] Expression vectors for use in the present invention are chimeric nucleic acid constructs (or expression vectors or cassettes), designed for operation in plants, with associated upstream and downstream sequences.

[0074] In general, expression vectors for use in practicing the invention include the following operably linked elements that constitute a chimeric gene: a promoter derived from a gene encoding a plant protein, operatively linked to a gene encoding a heterologous protein. The vector can include a promoter from the gene of a maturation-specific monocot plant storage protein, a first DNA sequence, operably linked to the promoter, encoding a monocot plant seed-specific signal sequence (such as an N-terminal leader sequence or a C-terminal trailer sequence) capable of targeting a polypeptide linked thereto to an endosperm cell, preferably an endosperm-cell organelle, such as a protein storage body, and a second DNA sequence, linked in translation frame with the first DNA sequence, encoding a cell culture media component. The signal sequence is preferably cleaved from the cell culture media component in the plant cell.

[0075] The chimeric gene, in turn, is typically placed in a suitable plant-transformation vector having (i) companion sequences upstream and/or downstream of the chimeric gene which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the desired plant host; (ii) a selectable marker sequence; and (iii) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region.

[0076] Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells. The promoter region is chosen to be regulated in a manner allowing for induction under seed-maturation conditions. In one aspect of this embodiment of the invention, the expression construct includes a promoter which exhibits specifically up-regulated activity during seed maturation. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, corn, millet, triticale or sorghum. Examples of such promoters include the maturation-specific promoter region associated with one of the following maturation-specific monocot plant storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

[0077] One embodiment of the present invention involves high-level expression of heterologous molecules that are components of cell culture media. These components can be expressed in a plant cell by a chimeric gene comprising the gene(s) encoding the desired component operably linked with an endogenous plant protein encoding gene. In a preferred embodiment the endogenous plant encoding gene is a rice seed storage protein gene for expression in a rice mature seed expression system.

[0078] The expressed polypeptide may be a multi-domain polypeptide with one domain being the heterologous or exogenous cell culture component and the other being the endogenous plant protein. At least one selective purification tag and/or at least one specific protease cleavage site may be provided for eventual release of the cell culture media component from the monocot seed storage protein carrier. For example, a strategic methionine or tryptophan residue providing a chemical cleavage site may be engineered in frame between the domains for release of the cell culture component from the endogenous plant protein. This technique is useful for cell culture media components that have protein portions or are proteins, for example lactoferrin, human serum albumin, and human growth factors.

[0079] Other selective protease cleavage sites include, but are not limited to enterokinase (ek), Factor Xa, thrombin, V8 protease, GENENASE™(a variant of subtilisin BPN'), α-lytic protease or tobacco etch virus protease. Alternatively, cleavage of the fusion protein could be performed via chemical cleaving agents such as cyanogen bromide or N-chlorosuccinimide.

[0080] The invention provides cell culture media components recombinantly produced in host monocot plant seed wherein the cell culture media component expressed comprises about 3% or greater of the total soluble protein in the monocot seed. Thus, for example, the yield of total soluble protein which comprises the cell culture media component targeted for production can be from about 3% to about 5%, from about 5% to about 10%, from about 10% to about 15%, from about 15% to about 20%, from about 20% to about 25%, and from about 25% to about 30% of the total soluble protein found in the recombinantly engineered production plant seed. Additionally, for example, the yield of total soluble protein which comprises the cell culture media component targeted for production can be about 3% or greater, about 5%

or greater, about 10% or greater, about 15% or greater, about 20% or greater, and about 25% or greater.

[0081] An embodiment of the present invention is a cell culture media, comprising at least one plant-produced heterologous protein as a cell culture media component. In some embodiments, the plant is a monocot plant, such as rice, barley, wheat, rye, corn, millet, triticale, or sorghum, preferably rice. The heterologous protein is a plant protein or a non-plant protein, preferably human, which may be selected in some embodiments from the group consisting of growth factors, lactoferrin, transferrin, serum albumin, insulin, growth hormone, and fractions thereof, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neutrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, or alpha-lactalbumin. In some embodiments of the present invention, the growth factors are preferably epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, or biologically active fragments thereof.

[0082] In some embodiments, the cell culture media is a reduced serum or serum-free medium. In other embodiments, the cell culture media is disclosed as a complete media, basal media, or basal media supplemented with a cell culture media component.

[0083] Another embodiment of the present invention is a method for producing a cell culture media, comprising producing at least one heterologous protein in a plant cell and combining the at least one plant-produced heterologous protein with a cell culture media.

[0084] Another embodiment of the present invention is a cell culture media produced by a process comprising producing at least one heterologous protein in a plant cell and combining at least one plant-produced heterologous protein with a cell culture media.

[0085] Another embodiment of the present invention is an improved cell culture media having one or more proteinaceous cell culture media components, the improvement comprising at least one plant-produced heterologous protein as a cell

culture media component, wherein the heterologous protein is produced by the following process:

a) transforming a plant cell with a chimeric gene comprising:

(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein; and

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein. In some embodiments, the heterologous protein is purified from the harvested seeds. In some embodiments, the heterologous protein constitutes at least about 3.0% of the total soluble protein in the harvested seeds.

[0086] Another embodiment of the present invention is a method for producing a cell culture media having at least one plant-produced heterologous protein as a cell culture media component, the method comprising:

a) transforming a plant cell with a chimeric gene comprising:

(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein;

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein;

c) harvesting the seeds from the plant; and

d) combining the heterologous protein with a cell culture media.

[0087] Another embodiment of the present invention is a method of producing monocot seeds, preferably rice grains, that can express the desired cell culture media component, comprising:

a) transforming a monocot plant cell with a chimeric gene comprising:
   (i) a promoter from the gene of a monocot seed storage protein;
   (ii) a first DNA sequence, operably linked to the promoter, encoding a monocot seed storage protein; and
   (iii) a second DNA sequence, operably linked to the promoter, encoding a cell culture media component,
   wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the cell culture media component;
b) growing a monocot plant from the transformed monocot plant cell for a time sufficient to produce seeds containing the cell culture media component; and
c) harvesting the seeds from the monocot plant.

[0088] Another aspect of the present invention is a method of utilizing monocot seed storage proteins as fusion protein carriers, comprising the above method.

[0089] The invention also includes a chimeric gene, comprising:
   (i) a promoter from the gene of a monocot seed storage protein;
   (ii) a first DNA sequence, operably linked to the promoter, encoding a monocot seed storage protein; and
   (iii) a second DNA sequence, operably linked to the promoter, encoding a cell culture media component;
   wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the cell culture media component.

[0090] The monocot seed storage protein may be at the N-terminal or C-terminal side of the cell culture media component in the fusion protein. It is preferred that the monocot seed storage protein by located at the N-terminal side of the cell culture media component.

[0091] Preferably, the monocot plant comprises rice, barley, wheat, rye, corn, millet, triticale, or sorghum. More preferably, the monocot plant is rice.

[0092] All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and indicated individually to be incorporated by reference in its entirety.

[0093] The following examples illustrate but are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Expression of recombinant human growth factors

[0094] In general, expression vectors were constructed using standard molecular biological techniques. The vectors contain a heterologous protein coding sequence for certain growth factors under the control of a rice tissue-specific promoter, as further described below.

[0095] The nucleotide sequence of the promoter and the nucleotide sequence of the signal peptide of the rice glutelin-1 (Gt1) gene were cloned based on the published Gt1 gene sequence. The nucleotide sequence of the promoter and the nucleotide sequence of the signal peptide of the rice globulin (Glb) gene were cloned based on the published Glb gene sequence.

A. Generation of human EGF expression vector

[0096] The human EGF gene was codon optimized as shown in Figure 1, and synthesized by Operon Technologies (CA, USA) (SEQ ID NO: 1). For expression of EGF in rice seeds, the codon optimized gene was operably linked to the rice endosperm specific glutelin (Gt1) promoter, Gt1 signal peptide and NOS terminator in pAPI303 (Figure 2), and to the rice endosperm specific globulin (Glb) promoter, Glb signal peptide and NOS terminator in pAPI270 (Figure 3). The transgenic plant expressing EGF was generated, and plant-generated recombinant EGF was detected, as shown in Figure 4 and as exemplified herein.

B. Generation of human IGF-I expression vector

[0097] The IGF-I gene was codon optimized as shown in Figure 5, and synthesized by Operon Technologies (CA, USA) (SEQ ID NO: 3). For expression of

IGF-I in rice seeds, the codon optimized gene was operably linked to the rice endosperm specific glutelin (Gt1) promoter, Gt1 signal peptide and NOS terminator in pAPI304 (Figure 7), and to the rice endosperm specific globulin (Glb) promoter, Glb signal peptide and NOS terminator in pAPI271 (Figure 6). The transgenic plant expressing IGF-I was regenerated, and plant-generated recombinant IGF-I was detected as shown in Figure 8 and as exemplified herein.

C. Generation of human ITF

[0098] The ITF gene was codon optimized as shown in Figure 9, and synthesized by Operon Technologies (CA, USA) (SEQ ID NO: 5). For expression of ITF in rice seeds, the codon optimized gene was operably linked to the rice endosperm specific glutelin (Gt1) promoter, Gt1 signal peptide and NOS terminator in pAPI307 (Figure 11), and to the rice endosperm specific globulin (Glb) promoter, Glb signal peptide and NOS terminator in pAPI269 (Figure 10). The transgenic plant expressing ITF was generated, and plant-generated recombinant ITF was detected as shown in Figure 12 and as exemplified herein.

D. Western blot analysis for all growth factors

[0099] Both untransformed (rice variety Taipei 309) and transgenic rice seeds (~10 pooled R1 seed from individual transgenic plants expressing either EGF, IGF-I or ITF) were ground in 1 ml of 0.35 M NaCl in phosphate buffered saline (PBS), pH 7.4, using an ice-cold mortar and pestle. The resulting extract was centrifuged at 14,000 rpm at 4°C for 10 min. Cleared supernatant was collected and ~20 ug of this soluble protein extract was re-suspended in sample loading buffer, and loaded onto a pre-cast 10-20% polyacrylamide tricine gel (Novex) and subjected to SDS-PAGE. After electrophoresis, the gel was blotted to a 0.45 µm nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in PBS pH 7.4 for 2 hrs followed by three washes with PBS for 10 min each. A primary rabbit polyclonal antibody prepared against EGF(Sigma), IGF-I (Sigma) or ITF (GI Company) was used at 1:2000 dilution in PBS. Bands were developed using goat anti-rabbit antibody coupled to the BCIP/NBT substrate system (Sigma). Results are shown in Figures 4, 8 and 12, respectively.

Example 2: Expression of recombinant human lactoferrin

A. An Expression Vector For Human Lactoferrin Expression In Transgenic Rice

[0100] Complete nucleotide sequence of human mammary gland lactoferrin was codon optimized and synthesized by Operon Technologies (CA, USA). Human milk lactoferrin gene (Genbank accession number: HSU07642) was re-synthesized with codons most frequently used in translation of rice seed proteins in order to obtain optimal level of expression (Figure 13). Although numbers of codons changed accounted for 22.46% of the entire sequence, amino acid composition remains identical to native human lactoferrin. The plasmid containing the codon-optimized gene was called Lac-ger. Lac-ger was digested with SmaI/XhoI and the fragment containing the lactoferrin gene was cloned into pAPI141 that was partially digested with NaeI and completely digested with XhoI. For expression of hLF in rice seeds, the codon-optimized gene was operably linked to the rice endosperm-specific glutelin (Gt1) promoter and NOS terminator. The resulting plasmid was designated pAPI164 (Figure 14).

B. Production system

[0101] Rice variety Taipei 309 (Oryza sativa, Japonica) was selected as the production system for recombinant human lactoferrin (rhLF) and transgenic rice events were generated by the particle bombardment of embryogenic rice calli with the plasmid pAPI164 and a companion marker plasmid containing the hygromycin phosphotransferase gene as a selectable marker. Fully developed, fertile rice plants were obtained by this procedure.

C. High Level Protein Expression of recombinant human lactoferrin in rice grain

[0102] Expression of recombinant human lactoferrin was under the control of the seed maturation-specific promoter Gt1. The high level expression of recombinant human lactoferrin is evident in Figure 15 as independent transgenic rice events were screened. Total soluble proteins from mature rice seed extracts were run on Laemli gels and stained with Coomassie blue to visualize the proteins. An ~80kD recombinant lactoferrin protein was obtained in all transgenic lines as indicated by the stained gel. Expression levels of recombinant human lactoferrin corresponded to 0.5% of seed weight. The stable expression of recombinant human lactoferrin was monitored for 10

generations. The expression level is maintained at approximately 0.5% of seed weight of brown rice (Figure 16).

Example 3: Expression of recombinant human serum albumin in rice grain

[0103] Protein sequences of human serum albumin (HSA) from various data bases were compared. The consensus sequence represented by accession number P02768 (Swiss-Prot) was used as a base for gene codon-optimization for suitable expression of human serum albumin in rice grain (Figure 17). Gene synthesis was carried out by Blue Heron (Seattle, WA) and the synthetic fragment was inserted into pUC based vector to create pUC-HSA. After confirmation of correct DNA and protein sequences, pUC-HSA was digested with MlyI and XhoI. The fragment containing codon-optimized HSA gene was inserted into pAPI405, which had been precut with NaeI and XhoI. Plasmid API405 was a derivative of pAPI141 which included Gt1 promoter, Gt1 signal sequence and a nos terminator.

[0104] Insertion of MlyI/XhoI fragment into pAPI405 resulted in pAPI504 (Figure 18). Plasmid API504 was then cleaved with HindIII and EcoRI. The HindIII/EcoRI fragment containing the entire expression cassette, Gt1 promoter, Gt1 signal sequence, codon-optimized HSA gene and nos terminator, was cloned into pJH2600 predigested with the same enzyme resulting in pAPI508 (Figure 19). Plasmid JH2600 was a shuttle vector between E coli and Agrobacterium. After DNA sequence verification, pAPI508 was moved into Agrobacterium AGL-1 for rice transformation. Plasmid API504 was also used via bombardment-based transformation following the procedure described previously. Upon transformation, transgenic plants were generated and were sent to greenhouse where transgenic R0 plants grew to maturity and set R1 seeds.

[0105] To monitor the expression of HSA in rice seeds, 10 R1 seeds from each R0 plant were extracted using 10 mL of extraction buffer (50 mM Tris-HCl pH 8.0. 150 mM NaCl). The supernatant was collected and the expression level from rice extracts was monitored by an ELISA (Bethyl Laboratories, Montgomery, TX). The results showed that the HSA expression level in rice transgenic seeds ranges from 0.01 to 0.85% of brown flour weight (0.1 to 8.5 grams/kg flour). The results of the eight events with highest expression levels are shown in the following table (Table 1).

Table 1. Recombinant HSA expression level in top eight events

Line Number	Express Level (g/kg flour)
508-3	5.5
508-17	8.0
508-71	8.7
508-73	4.0
508-77	8.5
508-83	8.5
508-101	3.0
508-113	4.0

Example 4: Purification of recombinant human lactoferrin as cell culture media component

[0106] To prepare cell culture media supplemented with recombinant human lactoferrin, recombinant human lactoferrin was purified from rice flour. Transgenic rice line (164-12) expressing high levels of rhLF was selected. This line, now named as LF164, was planted two generations per year, alternating field planting in summer and greenhouse planting in winter. For protein purification, paddy rice expressing rhLF was de-hulled (Rice Mill, PS-160, Rimac, FL), and then ground to flour (average particle size of 100 mesh) using a hammer mill (8WA, Schutte-Buffalo, NY).

[0107] Protein extraction from transgenic flour was performed by mixing two kg of rice flour and 20 L of extraction buffer (0.02 M sodium phosphate pH 6.5 and 0.3 M sodium chloride) in a 50 L tank for 1 h. At the end of the mixing period, the suspension was allowed to settle overnight or centrifuged at 3750 rpm. In both cases, the supernatant was filtered through a plate and frame filter press (Ertel Alsop, 8S, NY) using M-05 and M-70 cellulose/perlite-based filters (Ertel Alsop, NY), respectively.

[0108] The filtrate containing rhLF and other rice flour soluble proteins was loaded onto an ion exchange column for further purification. An INDEX 200/500 process column (Amersham Pharmacia Biotech, NJ) packed with SP-Sepharose fast

flow (Amersham Pharmacia Biotech, NJ) was used. The column was run at a linear flow rate of 150-200 cm/h. Packing, cleaning and testing of the resin bed was executed per manufacturer's instruction (HETP Test). The filtrate was loaded on to the resin at a linear velocity of 175 cm/h and washed with 0.02 M sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl until the A_{280} returned to baseline. Recombinant hLF was eluted using 20 mM sodium phosphate buffer (pH 6.5) containing 0.8 M NaCl. The washing and elution were performed at 200 cm/h and 150 cm/h, respectively.

[0109] A Centramate module (Pall Biopharmaceutical, MA) with 1 ft^2 50 kDa polyethersulfone (Pall Biopharmaceutical, MA) membrane was used for concentration and desalting (ultrafiltration) of eluted hLF. The filtration was performed at a cross flow rate of approximately 1.5 L/min and an average transmembrane pressure of 10 psig. The eluted rhLF was concentrated and desalted to a final volume of 0.25 L and then lyophilized dry. Usually, about 3 grams of purified recombinant human lactoferrin was recovered from one kilogram of transgenic rice flour.

[0110] The recombinant human lactoferrin purified from rice flour is approximately 50% saturated with iron (partial-lactoferrin). The 50% saturated recombinant human lactoferrin was then made >90% iron saturated by iron up taking treatment, resulting in holo-lactoferrin and was made <10% iron saturated by acid treatment to remove bound iron resulting in apo-lactoferrin. The purified lactoferrins (holo-, partial- and apo-) were used as cell culture media components.

Example 5: Cell culture media supplemented with recombinant human lactoferrin promotes cell growth

[0111] Recombinant human lactoferrin was added to minimal essential media (MEM) at various concentrations. This allows the test of growth factor effect of lactoferrin. The human colonic cancer cell line, HT-29, were maintained in MEM basic media containing 10% fetal calf serum (FCS). Lactoferrin samples were subsequently tested under serum-free conditions (MEM without supplementation of fetal calf serum). To assess the percentage of cells entering DNA synthesis, [^{3}H]-thymidine (2 µCi/well) was included twenty four hours after the addition of lactoferrin samples and cells were left for a further 24 h. For each condition, the stimulatory or inhibitory effect of the lactoferrin solutions was measured in duplicate in six separate wells. Cell viability,

determined by the ability to exclude 0.2% trypan blue, was greater than 90%. Proliferation assays reveal a typical 'bell shaped' dose response curve (Figure 20). Results demonstrated these responses to lactoferrin addition and show that maximal activity of lactoferrin was at a final concentration of 1 mg/ml, for all three forms of lactoferrin. Furthermore, of the three forms, the holo-lactoferrin showed the greatest proliferative activity (Figure 20).

[0112] To determine cell viability, HT-29 Cells were seeded in 24-well plates at 5 x 10^5 cells per well in MEM supplemented with 5% fetal calf serum (FCS); this resulted in an attached cell number of >95% per well 24h later (as assessed by trypsinization and direct counting using a Neubauer haemocytometer). At this time, the medium was changed in identically seeded wells to one containing MEM with 5 % FCS alone or MEM with 5% FCS plus EGF, or additional 5% FCS or various forms of Lactoferrin (i.e., apo-, partial- or holo-) at a concentration of 1 mg/ml for a further 48h. At the end of this period, cell numbers were assessed. To ensure that any cells present in the supernatant at this time were included in the total cell count, supernatants were collected, centrifuged at 13,000 rpm (12,000 g) for 5 min and added to the cells following trypsinization. Result shows that recombinant human lactoferrin derived from rice promote cell growth. There were twice as many cells in wells supplemented with holo-lactoferrin as that in wells without lactoferrin (Figure 21). Both thymidine incorporation and cell count experiments indicate that recombinant human lactoferrin from rice can be added as a component to cell culture media for fast cell growth (Figures 20 and 21).

Example 6: Cell culture media supplemented with recombinant human lactoferrin promotes hybridoma cell growth

[0113] Recombinant human holo-lactoferrin was added to serum free media at various concentrations ranging from 5 to 250 mg/ml for hybridoma cell culture. Since the recombinant human lactoferrin is iron-saturated, holo-lactoferrin can provide iron for cell growth. The hybridoma cell line AE1 (ATCC) was maintained in DMEM basic media containing 5% fetal bovine serum (FBS). Lactoferrin was tested under serum-free conditions (AFM6, KC Bio, Kansas) without supplementation of fetal bovine serum. The cells were subcultured from 5% FBS to serum free media over multiple passages. At

each subculture, the cells were analyzed for total cell count and viability. In order to demonstrate that recombinant human lactoferrin can replace transferrin derived from animal source, transferrin of equal concentration was added to AFM6 media as the control. The results demonstrated that the recombinant human holo-lactoferrin can be used to replace animal-derived transferrin in cell culture media for hybridoma cell growth.

[0114] To determine the ability of holo-lactoferrin to promote cell growth, AE1 cells were sub-cultured from media containing 5% FBS to serum free media in T25 stationary flasks. The serum free media was supplemented with either animal derived transferrin; which is commonly used in the industry, or recombinant human holo-lactoferrin (LACROMIN™). This allows for a direct comparison between recombinant human holo-lactoferrin and animal derived transferrin. Each protein test was performed in triplicate with seeding densities of 5 $X10^5$ cells per ml. To the subculture, the cells were collected by centrifugation and then seeded into fresh media with identical concentrations of either transferrin or recombinant human holo-lactoferrin (LACROMIN™). Each flask was analyzed on day 1 and day 3, following the subculture, for cell number and viability. An average cell count is given in Figure 22 for two passages. As can be seen, the cell viability and cell number when using recombinant human holo-lactoferrin are much higher than that without lactoferrin and equivalent to that of animal derived transferrin, indicating that plant-derived recombinant human holo-lactoferrin can promote hybridoma cell growth and replace animal derived transferrin.

[0115] The same analysis was carried out with another hybridoma cell line, L243. As can be seen in Table 2. LACROMIN™ (rhLF) can promote cell growth as as well as human transferrin (TF). L243 is also an IgG producing cell line. Samples were taken for IgG production. In medium containing LACROMIN™, more IgG is produced than in medium containing transferrin.

Table 2. Effect of human transferrin (TF) and recombinant human lactoferrin (rhLF) on hybridoma growth and monoclonal antibody production.

	TF/rhLF	Cell count ($X10^6$)		IgG level (ug/ml)	
Treatment	(ug/ml)	Mean	SD	Mean	SD
Control	0	0.39	0.18	15.78	5.63
TF	5	0.70	0.25	34.10	8.44
rhLF	5	0.63	0.10	43.58	8.24
TF	25	0.78	0.24	31.93	7.10
rhLF	25	0.81	0.20	36.10	7.54
TF	100	0.86	0.33	28.58	5.82
rhLF	100	0.78	0.26	29.73	4.28
5% FBS	0	0.98	0.12	23.88	5.50

Example 7: Purification and characterization of recombinant human serum albumin

[0116] To purify rHSA from rice grain, rice grain expressing rHSA was dehusked, ground to flour and mixed for 30 min. with rHSA extraction buffer (Sodium Acetate, pH 4.9). After clarification by filtration, the filtrate was loaded onto an anion-exchange column (Q-Sepharose; GE Healthcare) at a rate of approximately 150 cm/hr. Once loaded, the resin was then washed with equilibration buffer to remove any unbound protein. The eluted rHSA is approximately 90% pure as analyzed by SDS-PAGE. Blue-Sepharose (GE Healthcare) can then be used to increase the purity from 90% to approximately 98% based on scanning analysis of SDS-PAGE gels. The rHSA is then concentrated and desalted via ultrafiltration prior to freeze drying.

[0117] Purified rHSA was subjected to a range of biochemical and biophysical characterizations for comparison to pHSA (Plasma-derived HSA) (Table 3). These tests indicate that rHSA expressed in rice is equivalent to pHSA.

Table 3. Properties of rHSA as compared to pHSA

Property	pHSA	rHSA
N-terminal sequence	DAHKSE	DAHKSE
Glycosylation	None	None
SDS-PAGE/Western	~66 kDa	~66 kDa
Molecular mass (MALDI)	66.9-68.1 kDa	66.8-67.9 kDa
Isoelectric focusing point	pI 5.3	pI 5.3
Ligand binding	Yes	Yes
Thermal stability	Midpoint 65°C	Midpoint 65°C
Esterase activity	Yes	Yes
Protease sensitivity	Same as each other	Same as each other

Example 8: Cell culture media supplemented with recombinant human serum albumin promotes hybridoma cell growth in reduced -serum media.

[0118] The effectiveness of recombinant human serum albumin from rice (rHSA) to stimulate cell growth similar to plasma derived human serum albumin (pHSA) was tested. Basal medium DMEM supplemented with 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, 0.20 mg/ml ethanolamine and 2 mM Glutamax (Gibco) was used as common medium (CM). Hybridoma AE1 stocks in CM + 10% fetal bovine serum (FBS), were divided into four lineages: 1) CM +10% FBS as positive control 2) CM without HSA as negative control 3) CM + 100 mg/L rHSA as experimental, and 4) CM +100mg/L plasma-derived HSA (pHSA) as comparative control. Each lineage was then gradually adapted to 1% FBS (reduced serum) by sequential passage of $1x10^5$ cell/ml into medium containing 50% reduced FBS. At 1% FBS, each lineage was subsequently passaged 10 times to allow for full adaptation to growth under reduced-serum (1% FBS) conditions. Figure 23 shows cell growth curves of the 4 lineages during the 10th passage under CM +1%FBS. It was found that AE1 hybridoma cells grew much faster and reached a higher density in CM rHSA than in CM without any HSA (Figure 23). It was also found that cells grown in media with rHSA supplementation grew equivalently or better than cells grown in media with plasma derived HSA. This result indicates that rHSA from rice is functionally equivalent to pHSA in enhancing hybridoma cell growth in reduced-serum media.

Example 9: Cell culture media supplemented with recombinant human serum albumin promotes hybridoma cell growth in serum-free media.

[0119] In order to demonstrate that plant derived recombinant human serum albumin functions equivalently to albumin derived from animals to support cell growth, the growth of AE1 hybridoma cells in supplemented serum-free media (SFM) was tested. SFM was prepared that was supplemented with either recombinant human serum albumin or plasma derived human serum albumin at similar concentrations (100 mg/L). SFM without supplemental albumin was prepared as a negative control. This approach allows for direct comparison between plant derived recombinant human serum albumin (CELLASTIM™) and plasma derived human serum albumin in promoting cell growth. SFM consisted of DMEM/F12 basal media supplemented with 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, 0.20 mg/ml ethanolamine and 2 mM Glutamax (Gibco). To reach serum-free growth conditions, AE1 Hybridoma cells (ATCC # HB-72) were first adapted to SFM without FBS by a serial adaptation procedure. Briefly, AE1 grown in SFM/10% FBS supplemented with recombinant human serum albumin or plasma derived human serum albumin or no HSA, were successively sub-cultured at 1x 10^5 cells/ml into SFM with 50% reduced serum from the previous culture. The SFM/0.25% FBS cultures were subcultured directly into SFM without FBS. Six successive subcultures followed in SFM to allow cells to fully adapt to the respective SFM without FBS.

[0120] AE1 hybridoma cells grown for 6 passages in SFM containing 100 mg/L recombinant human serum albumin or 100 mg/L plasma derived human serum albumin or no HSA were sub-cultured 6-well plates at 1x10^5 cells/ml. The number of viable cells and percent viability was determined daily to examine growth kinetics of the cultures. The subcultures were performed in duplicate to ensure an accurate viable cell count and analysis. Figure 24 shows that AE1 hybridoma cell grew faster and that the maximum number of viable cells was higher in medium supplemented with plant derived recombinant human serum albumin and equivalent to that of plasma derived human serum albumin. Thus, this example shows that plant derived recombinant human serum albumin can promote cell growth in serum-free media and replace animal derived albumin for use in tissue culture.

[0121] The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

IN THE CLAIMS:

1. A cell culture media, comprising at least one plant-produced heterologous protein as a cell culture media component.

2. The cell culture media of claim 1, wherein the plant is a monocot plant.

3. The cell culture media of claim 2, wherein the monocot plant is rice, barley, wheat, rye, corn, millet, triticale, or sorghum.

4. The cell culture media of claim 3, wherein the monocot plant is rice.

5. The cell culture media of claim 1, wherein the plant is a dicot plant.

6. The cell culture media of claim 1, wherein the heterologous protein is a plant protein.

7. The cell culture media of claim 1, wherein the heterologous protein is a non-plant protein.

8. The cell culture media of claim 7, wherein the non-plant protein is an animal protein.

9. The cell culture media of claim 7, wherein the non-plant protein is a human protein.

10. The cell culture media of claim 1, wherein the protein is selected from the group consisting of growth factors, lactoferrin, transferrin, serum albumin, insulin, growth hormone, and fractions thereof, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neutrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, or alpha-lactalbumin.

11. The cell culture media of claim 10, wherein the growth factors are epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, or biologically active fragments thereof.

12. The cell culture media of claim 1, wherein the cell culture media is a reduced serum, serum-free medium, or chemically defined media.

13. The cell culture media of claim 1, wherein the cell culture media is a complete media, basal media, or basal media supplemented with a cell culture media component.

14. A method for producing an enhanced cell culture media, comprising producing at least one heterologous protein in a plant cell and combining the at least one plant-produced heterologous protein with a cell culture media.

15. A cell culture media produced by a process comprising producing at least one heterologous protein in a plant cell and combining the at least one plant-produced heterologous protein with a cell culture media.

16. In a cell culture media having one or more proteinaceous cell culture media components, the improvement comprising at least one plant-produced heterologous protein as a cell culture media component, wherein the heterologous protein is produced by the following process:

a) transforming a plant cell with a chimeric gene comprising:

(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a signal sequence of a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein; and

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein.

17. The method of claim 17, further comprising purifying the heterologous protein from the harvested seeds.

18. The method of claim 17, wherein the heterologous protein constitutes at least about 3.0% of the total soluble protein in the harvested seeds.

19. A method for producing a cell culture media having at least one plant-produced heterologous protein as a cell culture media component, the method comprising:
a) transforming a plant cell with a chimeric gene comprising:

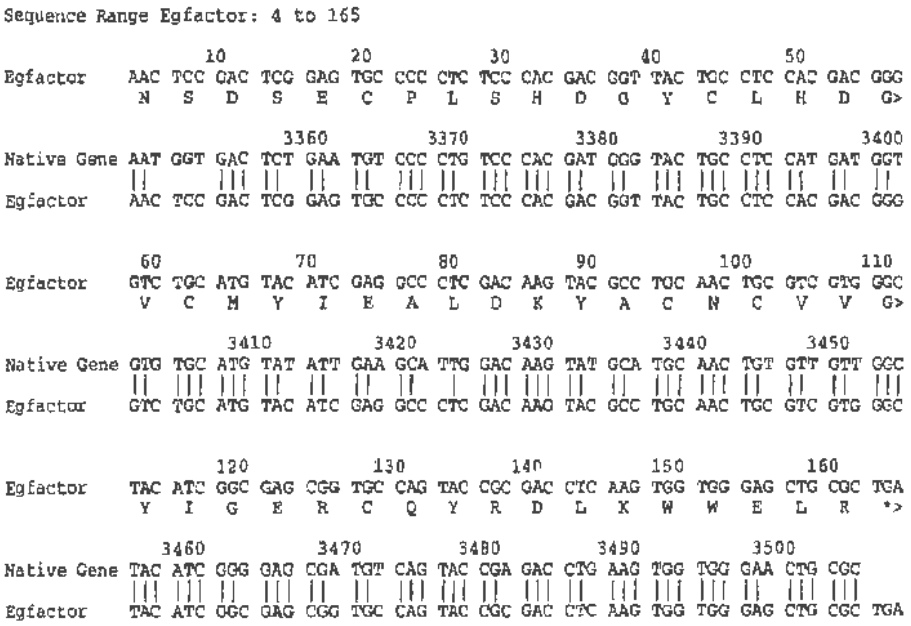
(i) a promoter from the gene of a seed storage protein;

(ii) a first DNA sequence, operably linked to the promoter, encoding a signal sequence of a seed storage protein, and

(iii) a second DNA sequence, operably linked to the promoter, encoding the heterologous protein, wherein the first and second DNA sequences are linked in translation frame and together encode a fusion protein comprising the storage protein and the heterologous protein;

b) growing a plant from the transformed plant cell for a time sufficient to produce seeds containing the heterologous protein;
c) harvesting the seeds from the plant; and
d) combining the heterologous protein with a cell culture media.

Figure 1. Comparison of Codon-optimized (egfactor) and Native Genes for the Epidermal Growth Factor (EGF)



Epidermal Growth Factor

Number of codons in mature peptide:	53
Number of codons changed:	27 (51%)
Number of nucleotides changed:	30 (19%)

Figure 2. Plasmid map of pAPI303 containing a chimeric gene for expressing human EGF under the control of the rice glutelin promoter in transgenic rice seeds.

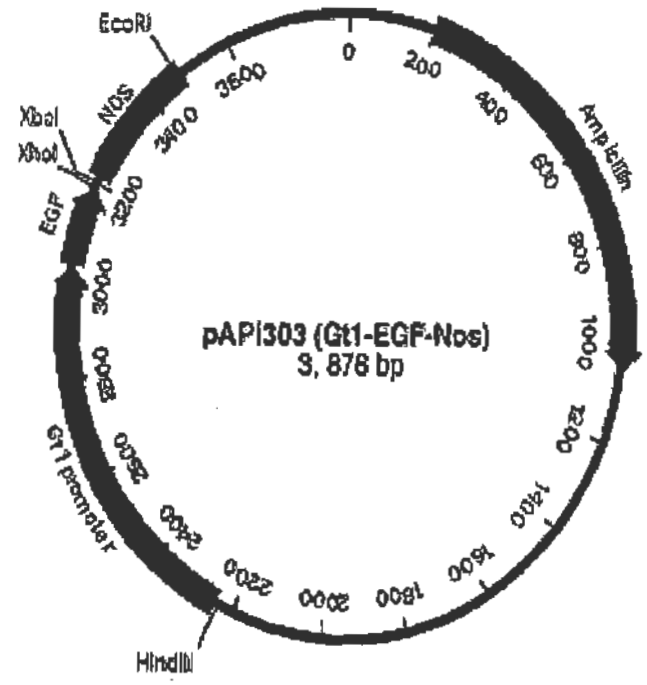


Figure 3. Plasmid map of pAPI270 containing a chimeric gene for expressing human EGF under the control of the rice globulin promoter in transgenic rice seeds.

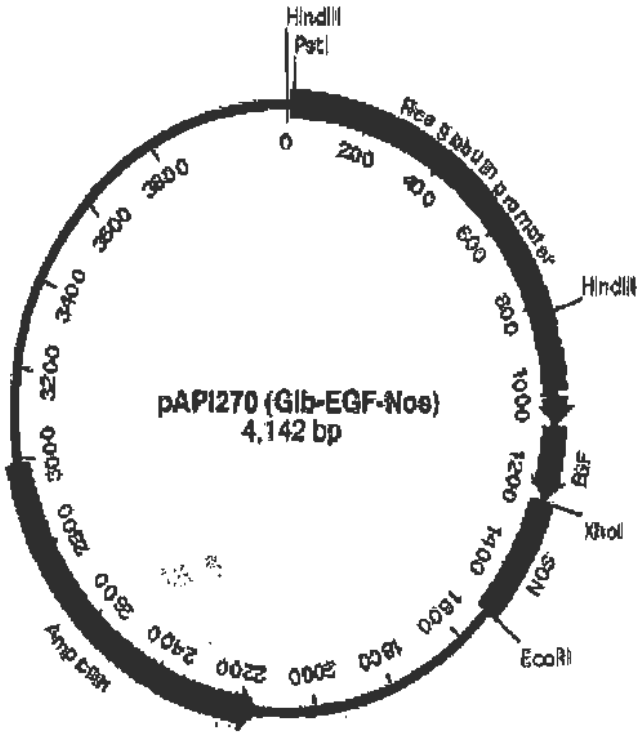


Figure 4. Western blot analysis of recombinant human epidermal growth factor (EGF) expressed in transgenic rice seeds.

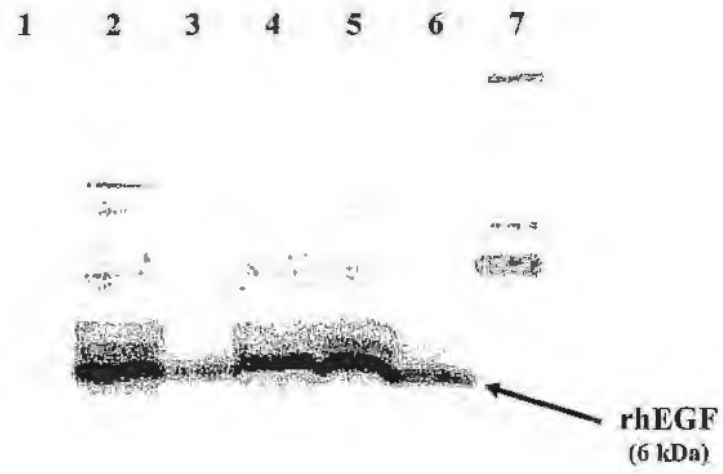
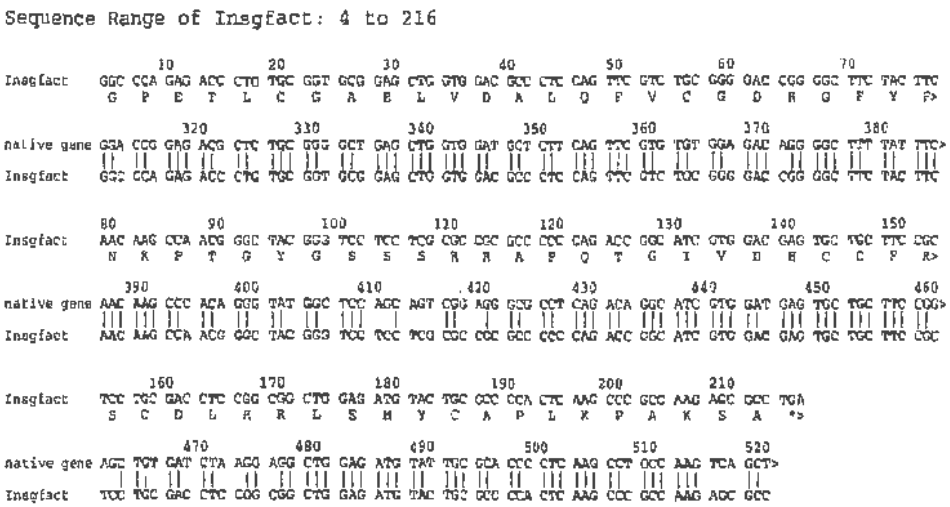


Figure 5. Comparison of Codon-optimized (Insgfact) and Native Genes for the Insulin-like Growth Factor (IGF)



Insulin-like Growth Factor

Number of codons in mature peptide: 70
Number of codons changed: 40 (57%)
Number of nucleotides changed: 47 (22%)

Figure 6. Plasmid map of pAPI271 containing a chimeric gene for expressing human IGF-1 under the control of the rice globulin promoter in transgenic rice seeds.

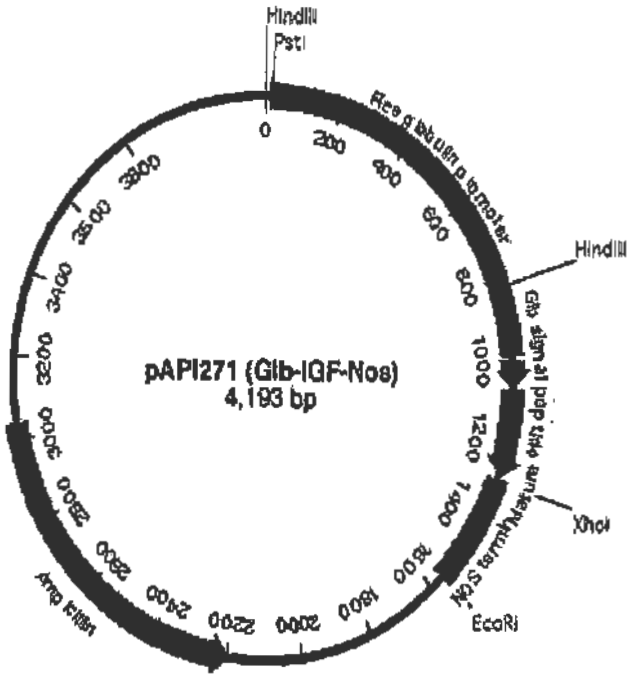


Figure 7. Plasmid map of pAPI304 containing a chimeric gene for expressing human IGF-1 under the control of the rice glutelin promoter in transgenic rice seeds.

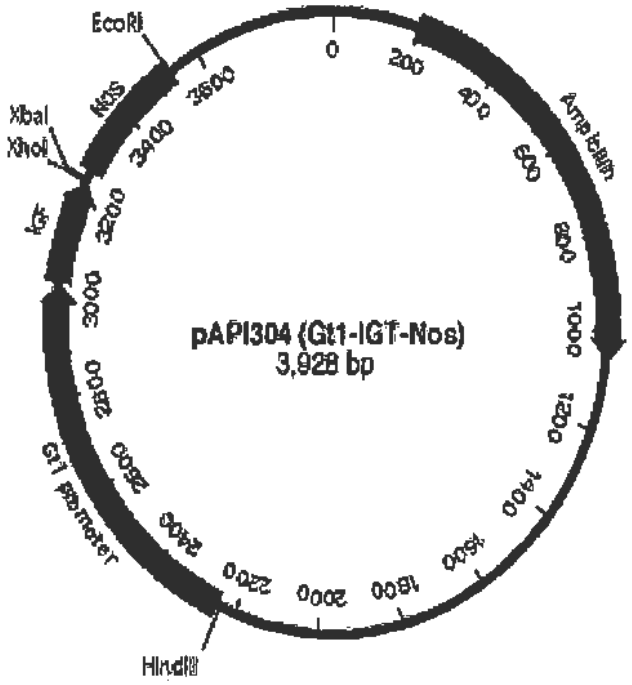
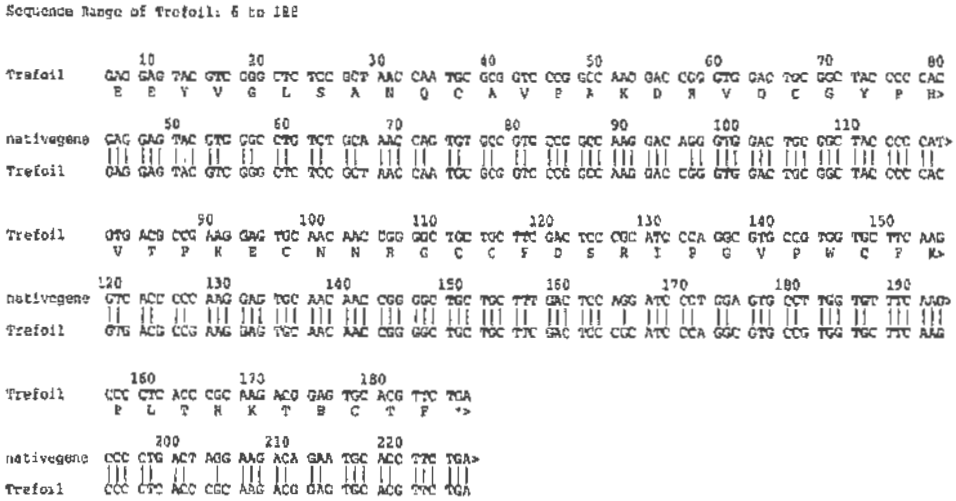


Figure 8. Western blot analysis of recombinant human insulin-like growth factor (IGF-1) expressed in transgenic rice seeds.



Figure 9. Comparison of Codon-optimized (Trefoil) and Native Genes for the Human Trefoil Peptide



Human Trefoil Peptide

Number of codons in mature peptide: 60
Number of codons changed: 26 (43%)
Number of nucleotides changed: 28 (15%)

Figure 10. Plasmid map of pAPI269 containing a chimeric gene for expressing human ITF under the control of the rice globulin promoter in transgenic rice seeds.

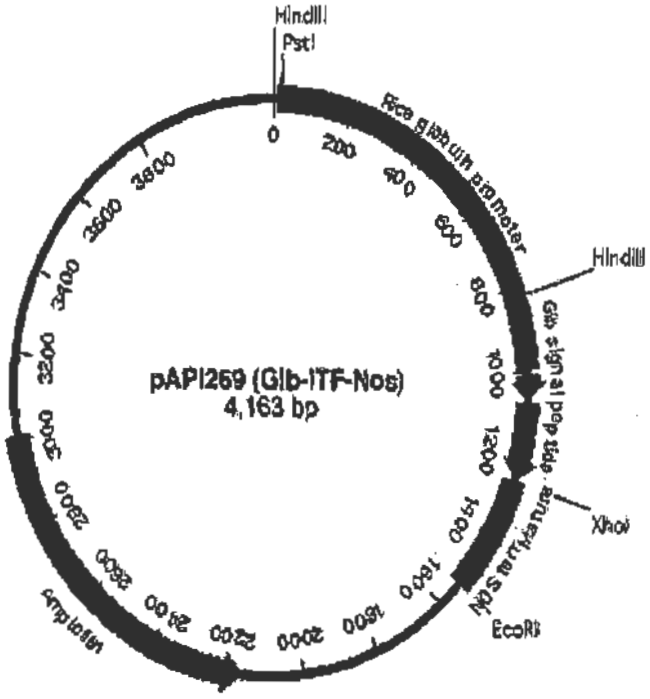


Figure 11. Plasmid map of pAPI307 containing a chimeric gene for expressing human ITF under the control of the rice glutelin promoter in transgenic rice seeds.

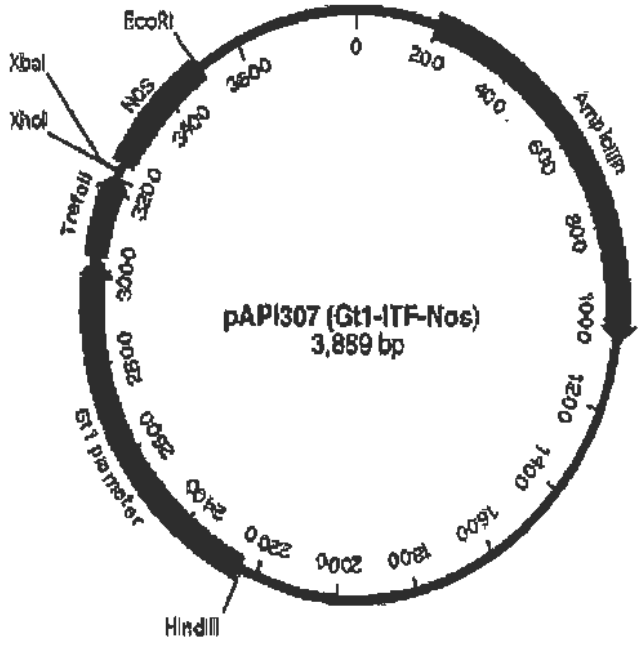


Figure 12. Western blot analysis of recombinant human intestinal trefoil factor (ITF) expressed in transgenic rice seeds.

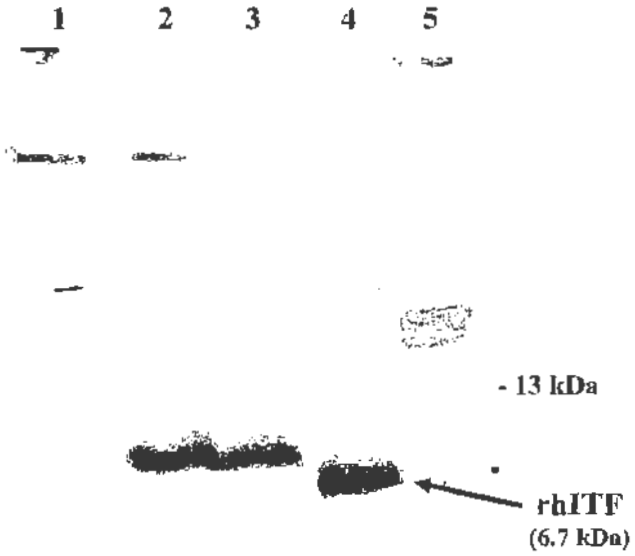
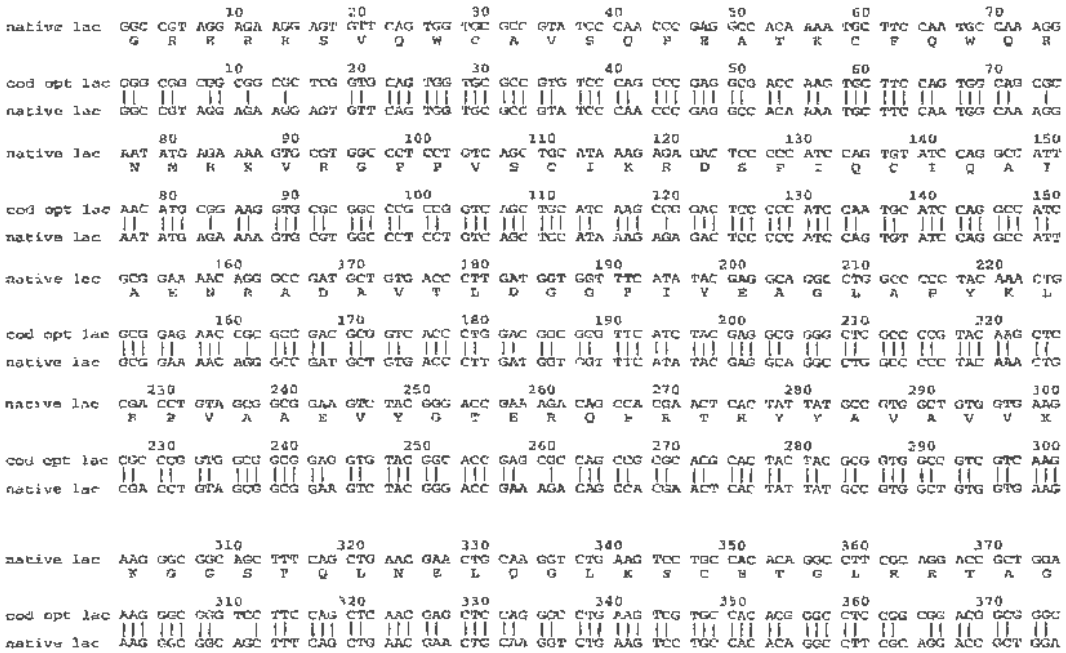
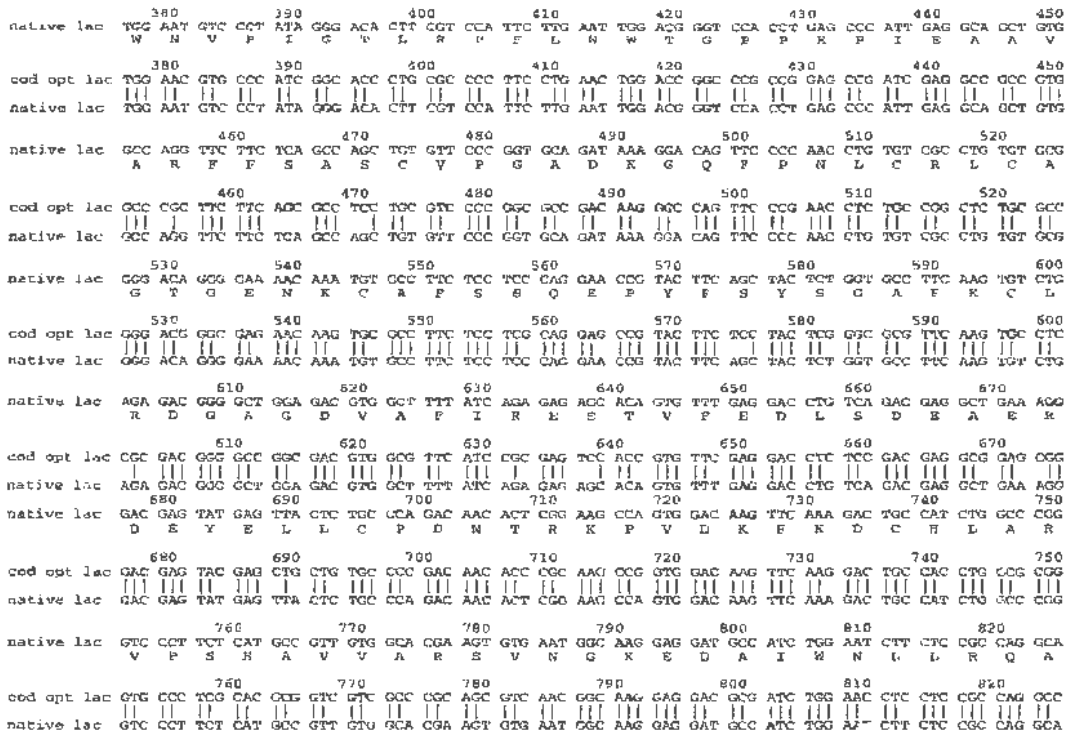
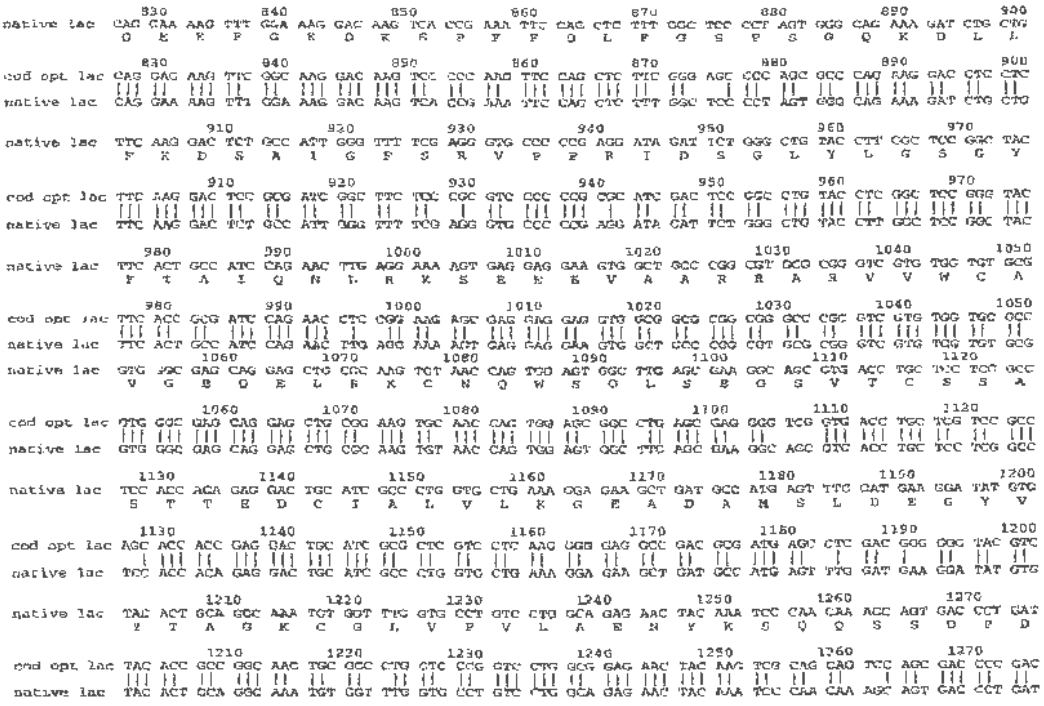
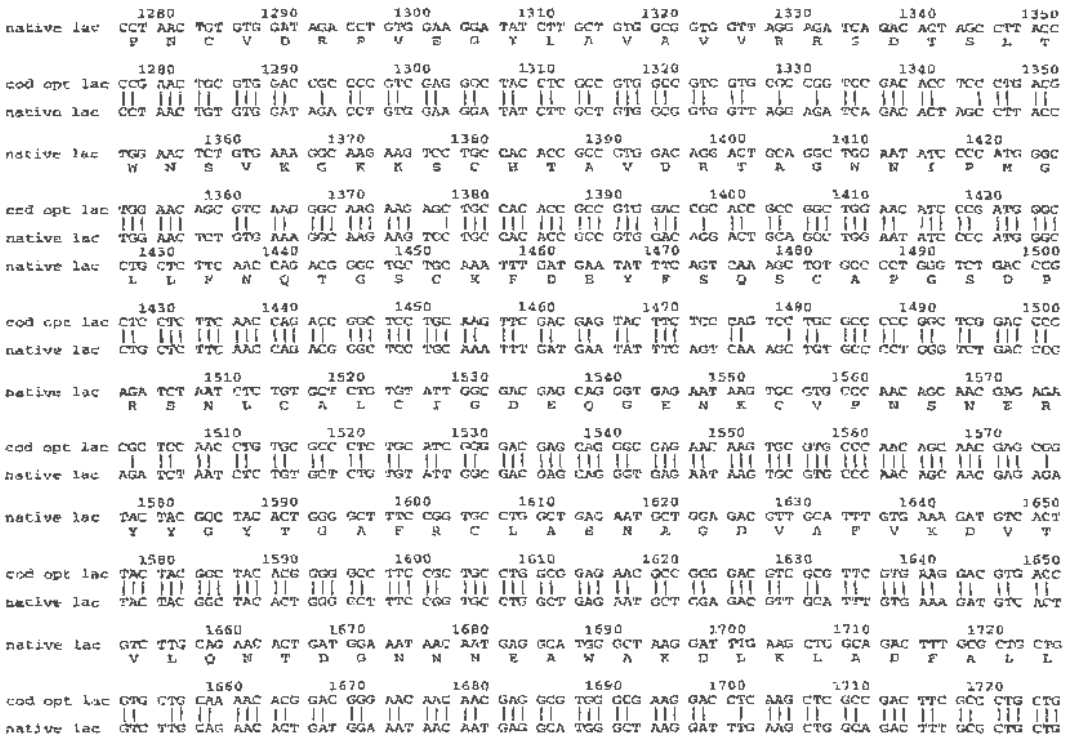


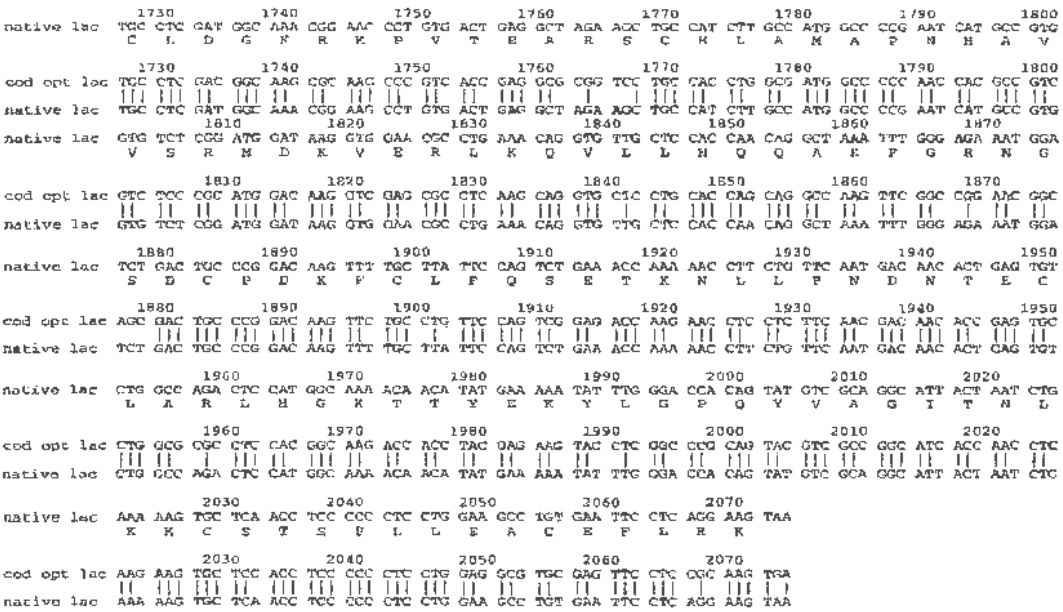
Figure 13. Codon-optimized Human Lactoferrin Gene and its Alignment to its Native Form











2079 = Total number of nucleotides

467 = Number of nucleotides changed

693 = Total Number of codons

413 = Number of codons changed

WO 2007/027742 PCT/US2006/025195

Figure 14: Plasmid map of pAPI164

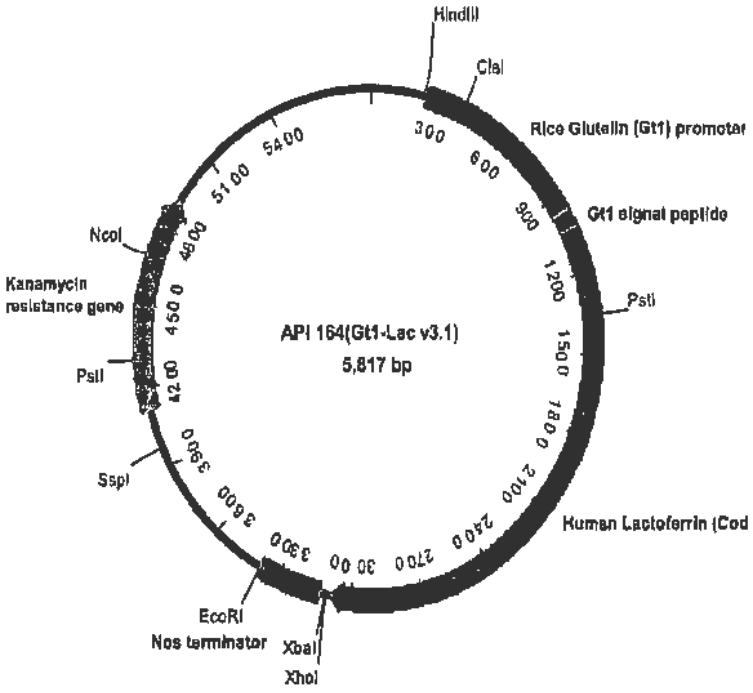


Figure 15: Recombinant human lactoferrin expressed in transgenic rice grains

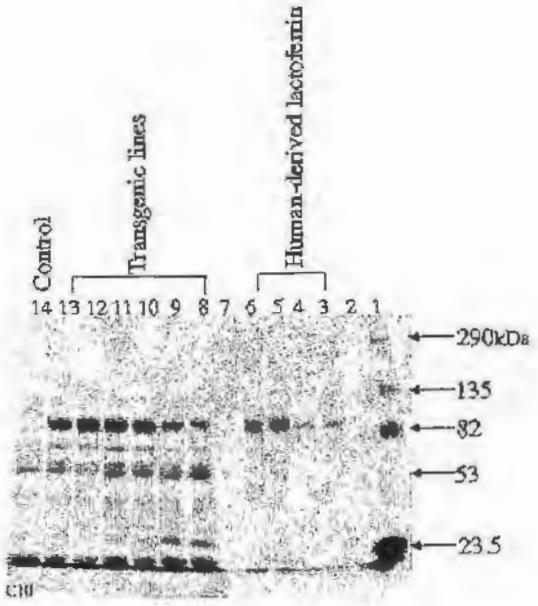


Figure 16. Stable expression of rhLF in transgenic rice grains from R_2 through R_{10} generations.

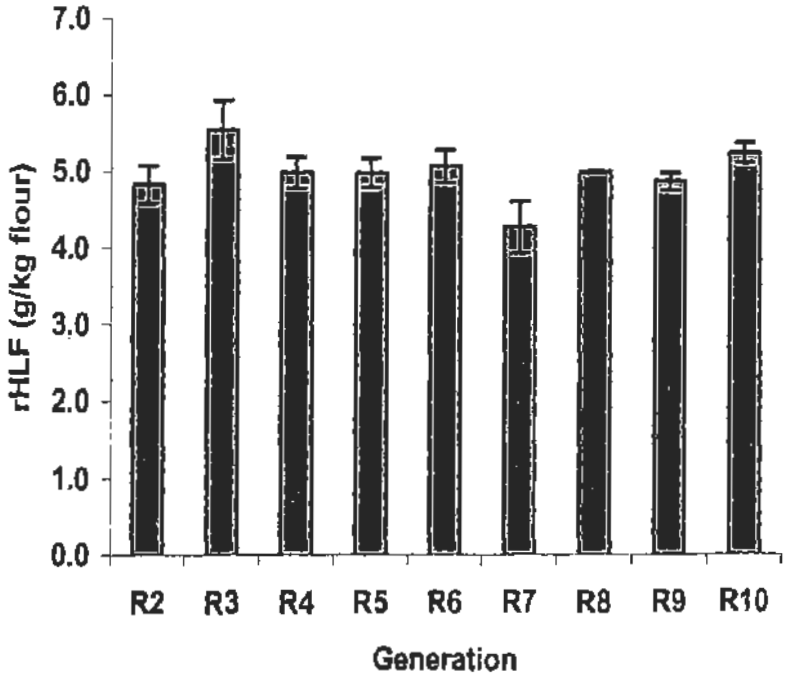
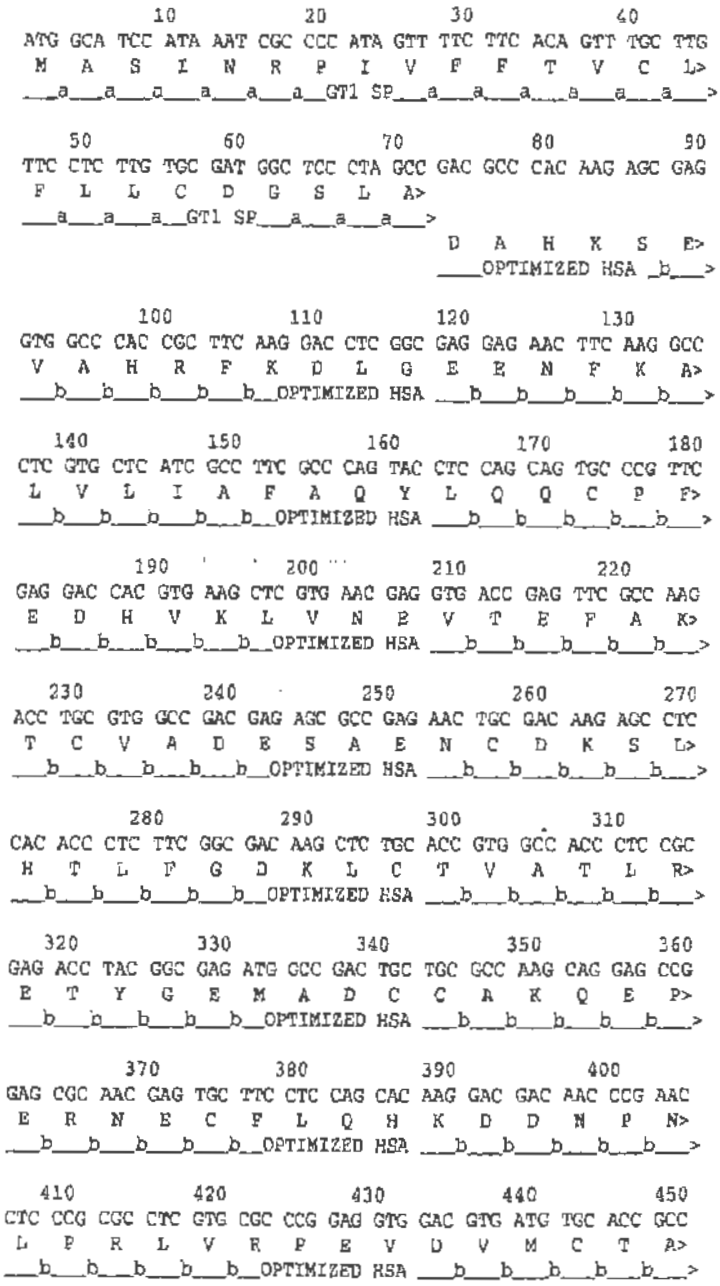


Figure 17 DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human serum albumin (OPTIMIZED HSA)



```
          460           470           480           490
TTC CAC GAC AAC GAG GAG ACC TTC CTC AAG AAG TAC CTC TAC GAG
 F   H   D   N   E   E   T   F   L   K   K   Y   L   Y   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   500           510           520           530           540
ATC GCC CGC CGC CAC CCG TAC TTC TAC GCC CCG GAG CTC CTC TTC
 I   A   R   R   H   P   Y   F   Y   A   P   E   L   L   F>
___b___b___b___b___b___OPTIMIZED HSA ___b___b___b___b___b___>

          550           560           570           580
TTC GCC AAG CGC TAC AAG GCC GCC TTC ACC GAG TGC TGC CAG GCC
 F   A   K   R   Y   K   A   A   F   T   E   C   C   Q   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   590           600           610           620           630
GCC GAC AAG GCC GCC TGC CTC CTC CCG AAG CTC GAC GAG CTC CGC
 A   D   K   A   A   C   L   L   P   K   L   D   E   L   R>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          640           650           660           670
GAC GAG GGC AAG GCC TCC AGC GCC AAG CAG CGC CTC AAG TGC GCC
 D   E   G   K   A   S   S   A   K   Q   R   L   K   C   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   680           690           700           710           720
AGC CTC CAG AAG TTC GGC GAG CGC GCC TTC AAG GCC TGG GCC GTG
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___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

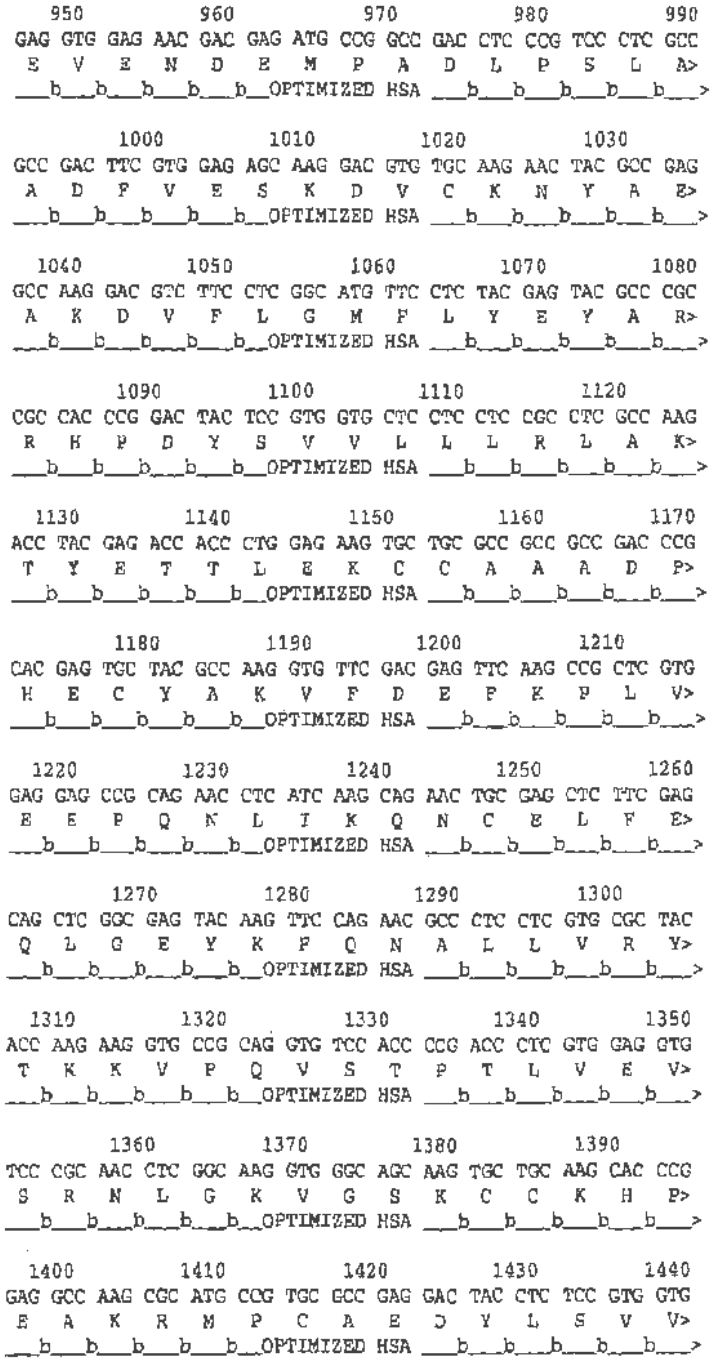
          730           740           750           760
GCC CGC CTC AGC CAG CGC TTC CCG AAG GCC GAG TTC GCC GAG GTG
 A   R   L   S   Q   R   F   P   K   A   E   F   A   E   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   770           780           790           800           810
TCC AAG CTC GTG ACC GAC CTC ACC AAG GTG CAC ACC GAG TGC TGC
 S   K   L   V   T   D   L   T   K   V   H   T   E   C   C>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          820           830           840           850
CAC GGC GAC CTC CTG GAG TGC GCC GAC GAC CGC GCC GAC CTC GCC
 H   G   D   L   L   E   C   A   D   D   R   A   D   L   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

   860           870           880           890           900
AAG TAC ATC TGC GAG AAC CAG GAC AGC ATC TCC AGC AAG CTC AAG
 K   Y   I   C   E   N   Q   D   S   I   S   S   K   L   K>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

          910           920           930           940
GAG TGC TGC GAG AAG CCG CTC CTG GAG AAG TCC CAC TGC ATC GCC
 E   C   C   E   K   P   L   L   E   K   S   H   C   I   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
```


950 960 970 980 990
GAG GTG GAG AAC GAC GAG ATG CCG GCC GAC CTC CCG TCC CTC GCC
E V E N D E M P A D L P S L A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1000 1010 1020 1030
GCC GAC TTC GTG GAG AGC AAG GAC GTG TGC AAG AAC TAC GCC GAG
A D F V E S K D V C K N Y A E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1040 1050 1060 1070 1080
GCC AAG GAC GTC TTC CTC GGC ATG TTC CTC TAC GAG TAC GCC CGC
A K D V F L G M F L Y E Y A R>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1090 1100 1110 1120
CGC CAC CCG GAC TAC TCC GTG GTG CTC CTC CTC CGC CTC GCC AAG
R H P D Y S V V L L L R L A K>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1130 1140 1150 1160 1170
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T Y E T T L E K C C A A A D P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
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H E C Y A K V F D E F K P L V>
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E E P Q N L I K Q N C E L F E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
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CAG CTC GGC GAG TAC AAG TTC CAG AAC GCC CTC CTC GTG CGC TAC
Q L G E Y K F Q N A L L V R Y>
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T K K V P Q V S T P T L V E V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1360 1370 1380 1390
TCC CGC AAC CTC GGC AAG GTG GGC AGC AAG TGC TGC AAG CAC CCG
S R N L G K V G S K C C K H P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
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E A K R M P C A E D Y L S V V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

```
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 L   N   Q   L   C   V   L   H   E   K   T   P   V   S   D>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1490         1500         1510         1520         1530
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 R   V   T   K   C   C   T   E   S   L   V   N   R   R   P>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1540         1550         1560         1570
TGC TTC TCC GCC CTG GAG GTC GAC GAG ACC TAC GTC CCG AAG GAG
 C   F   S   A   L   E   V   D   E   T   Y   V   P   K   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1580         1590         1600         1610         1620
TTC AAC GCC GAG ACC TTC ACC TTC CAC GCC GAC ATC TGC ACC CTC
 F   N   A   E   T   F   T   F   H   A   D   I   C   T   L>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1630         1640         1650         1660
TCC GAG AAG GAG CGC CAG ATC AAG AAG CAG ACC GCC CTC GTC GAG
 S   E   K   E   R   Q   I   K   K   Q   T   A   L   V   E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1670         1680         1690         1700         1710
CTC GTG AAG CAC AAG CCG AAG GCC ACC AAG GAG CAG CTC AAG GCC
 L   V   K   H   K   P   K   A   T   K   E   Q   L   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1720         1730         1740         1750
GTG ATG GAC GAC TTC GCC GCC TTC GTG GAG AAG TGC TGC AAG GCC
 V   M   D   D   F   A   A   F   V   E   K   C   C   K   A>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

  1760         1770         1780         1790         1800
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 D   D   K   E   T   C   F   A   E   E   G   K   K   L   V>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>

         1810         1820         1830
GCC GCC AGC CAG GCC GCC CTC GGC CTC TGA
 A   A   S   Q   A   A   L   G   L   *>
___b___b____OPTIMIZED HSA _b___b___b___>
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Figure 18. Plasmid map of pAPI504

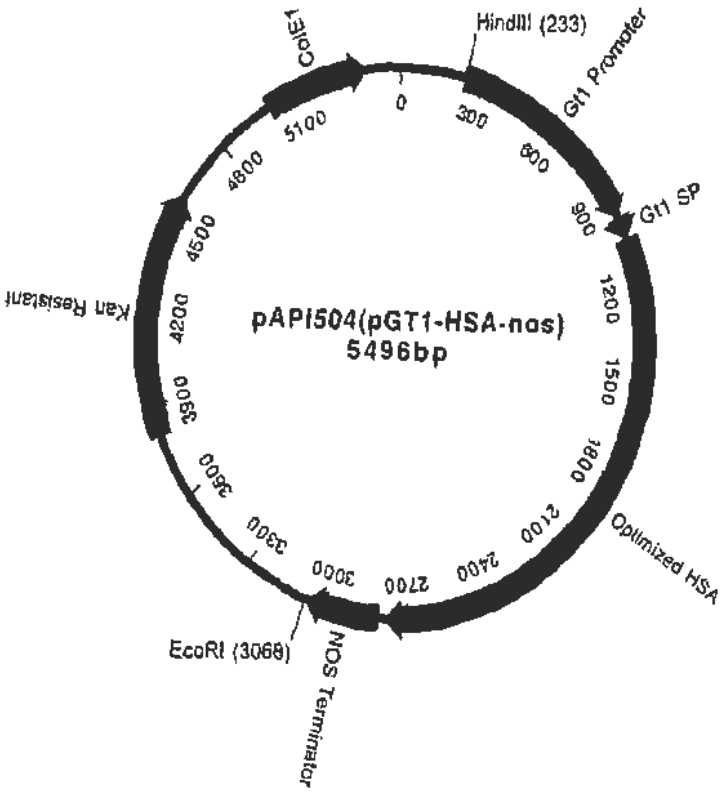


Figure 19. Plasmid map of pAPI508

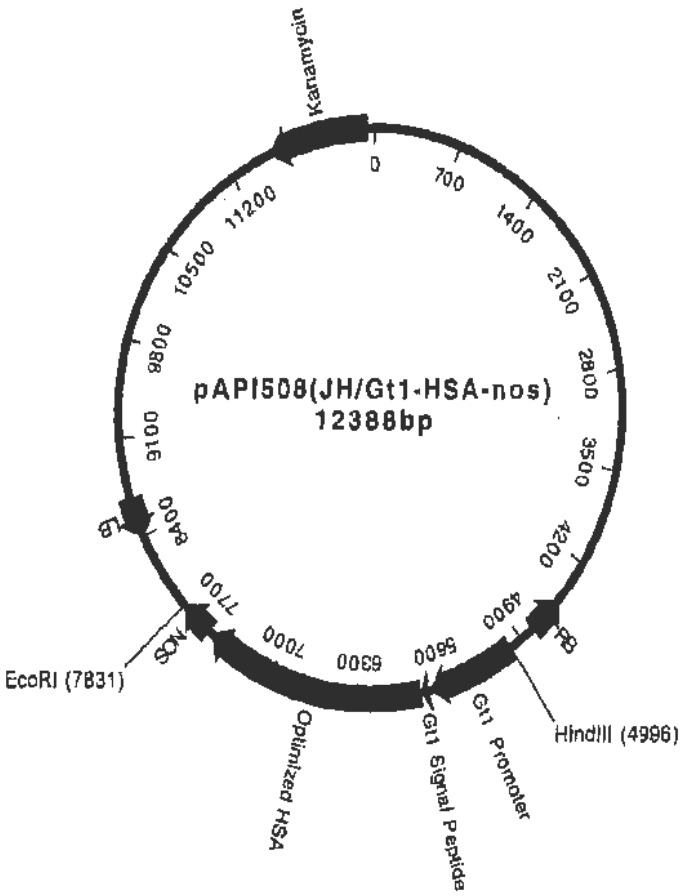


Figure 20 Impact of recombinant human lactoferrin on DNA replication of HT29 cells

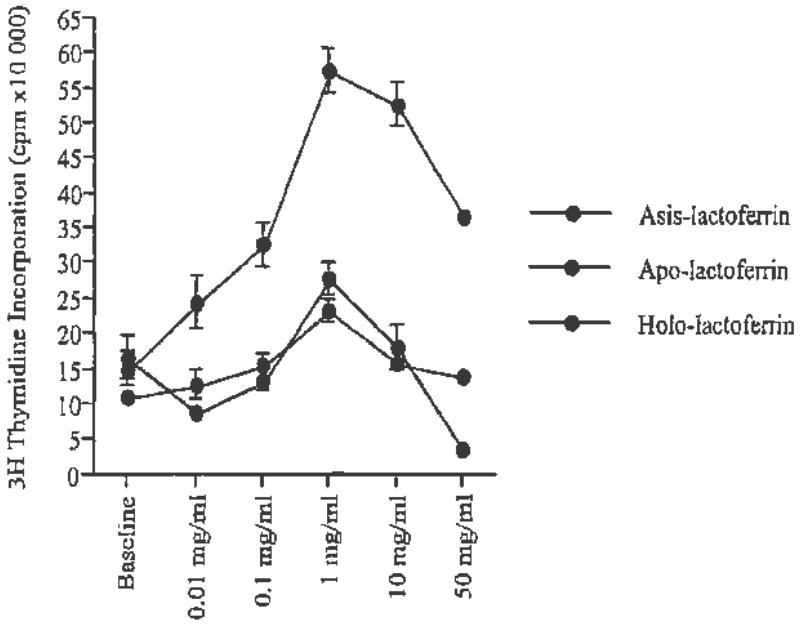


Figure 21. Cell culture media supplemented with recombinant human lactoferrin expressed in rice grain promotes cell growth and increases the cell number.

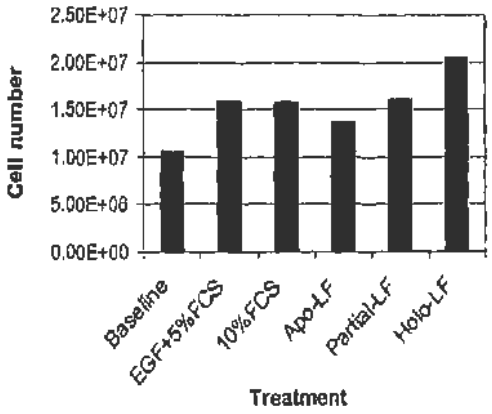


Figure 22. Cell culture media supplemented with recombinant human lactoferrin (Lacromin™) expressed in rice grain promotes hybridoma cell growth and increases hybridoma cell number.

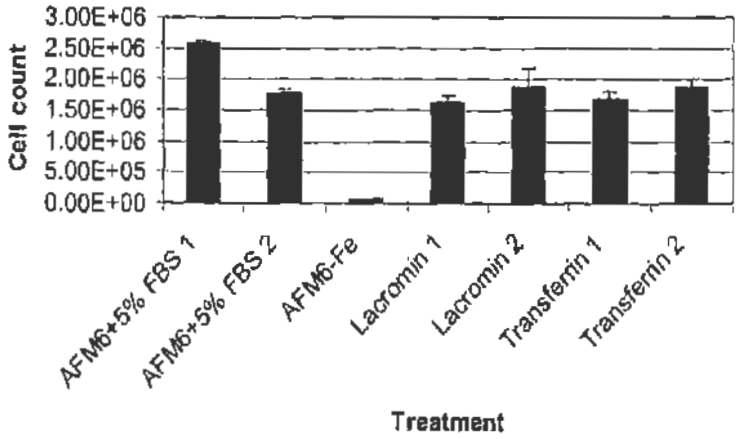


Figure 23 Effect of recombinant human serum albumin (rHSA) and plasma-derived human serum albumin (pHSA) on the growth of hybridoma AE1 cells in common medium (DMEM containing reduced 1% FBS) (CM). Cells were gradually adapted to CM with either 1)pHSA 2)nHSA or 3) no HSA and maintained for 10 passages to obtain a stable phenotype. On the 10th passage cells were seeded at $1x10^5$ cell/ml on day 0 and cell numbers were counted daily for four days.

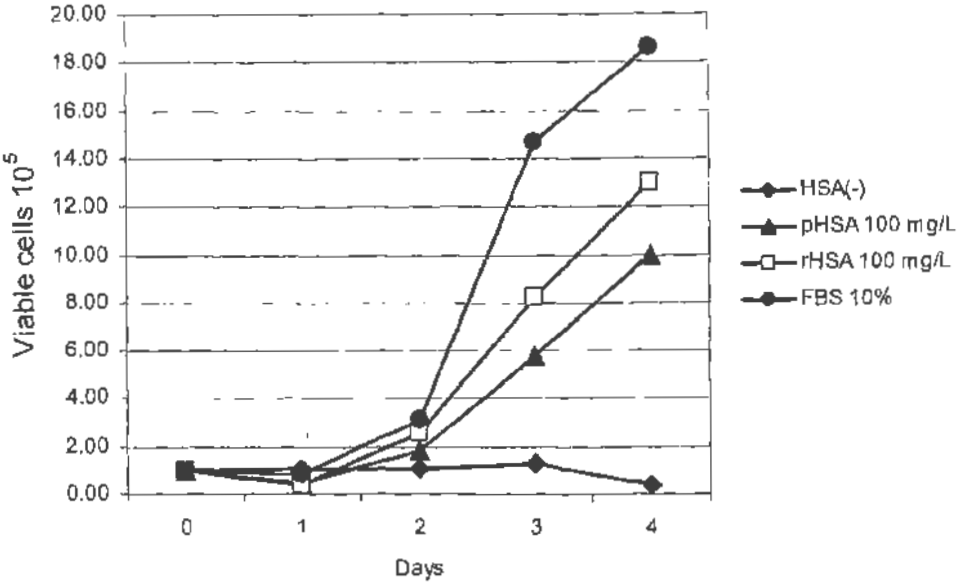
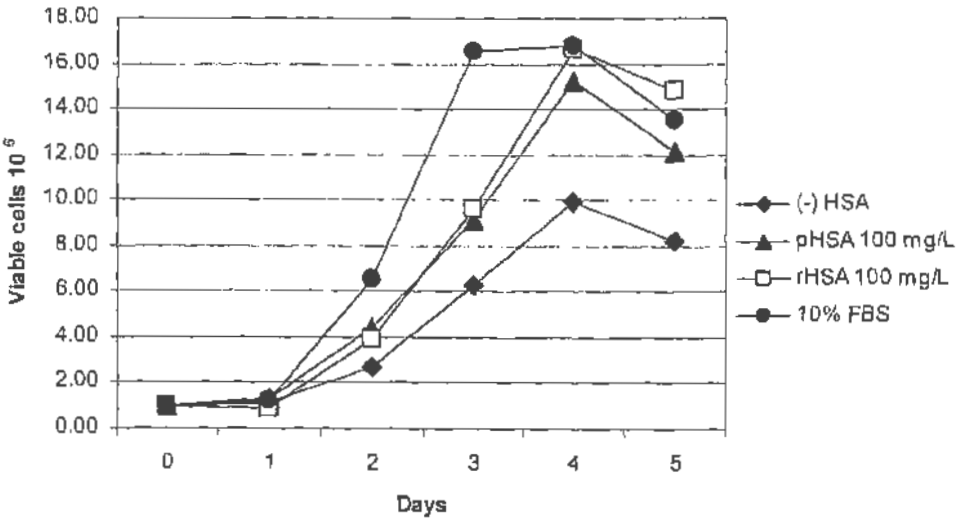


Figure 24. Effect of recombinant human serum albumin (rHSA) and plasma-derived human serum albumin (pHSA) on the growth of hybridoma AE1 cells in DMEM/F12 serum-free medium (SFM). Cells were gradually adapted to SFM with either 1) pHSA 2)rHSA or 3) no HSA and maintained for 6 passages to obtain a stable phenotype. On the 7th passage cells were seeded at $1x10^5$ cell/ml on day 0 and viable cell numbers were counted daily for 5 days.



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Group Art Unit No.: 1656
Filed: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. HOLLAND

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDMENT

In response to the Office Action dated October 16, 2018, amendment and reconsideration of the above-referenced application is requested.

Amendments to the Drawings begin on page 2.

Amendments to the Specification begin on page 3.

Claim Amendments begin on page 4.

Remarks begin on page 6.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Group Art Unit No.: 1656
Filed: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. HOLLAND

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

SEQUENCE STATEMENT

In response to the Office Action, please replace the original Sequence Listing with the Sequence Listing text file submitted herewith. The replacement Sequence Listing has been corrected to add the sequences originally presented in the drawing figures (but not separately listed in the Sequence Listing). No other changes have been made.

In accordance with 37 CFR 1.821, the sequence listing information recorded in computer readable form is identical to the sequence listing information written in the figures as filed, and the revisions to the Sequence Listing include no new matter. Any fee which is due in connection with this should be applied against Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Seymour Cook/
By_____
Crissa A. Seymour Cook, Reg. No. 64,054
HOVEY WILLIAMS LLP
10801 Mastin Boulevard, Suite 1000
Overland Park, KS 66210
913-647-9050

ATTORNEYS FOR APPLICANTS

PTO/AIA/22 (03-13)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 50091-CNT
Application Number 15/188,478	Filed June 21, 2016
For CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	
Art Unit 1656	Examiner Paul J. Holland

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☑	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☑ Applicant asserts small entity status. See 37 CFR 1.27

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed

☐ Payment by credit card. Form PTO-2038 is attached.

☑ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 190522.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☑ attorney or agent of record. Registration number 64054

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____

/Crissa A. Cook/ — Signature
March 18, 2019 — Date
Crissa A. Cook — Typed or printed name
913-647-9050 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*

☑ * Total of 1 forms are submitted

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

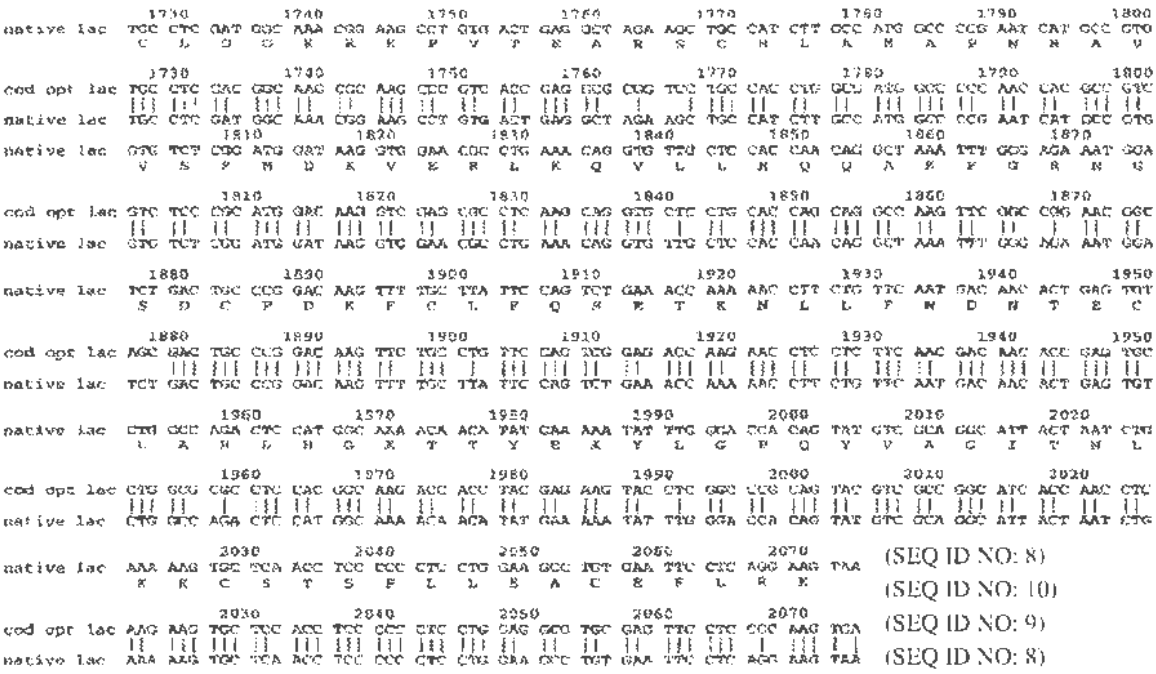
Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

FIG 1D



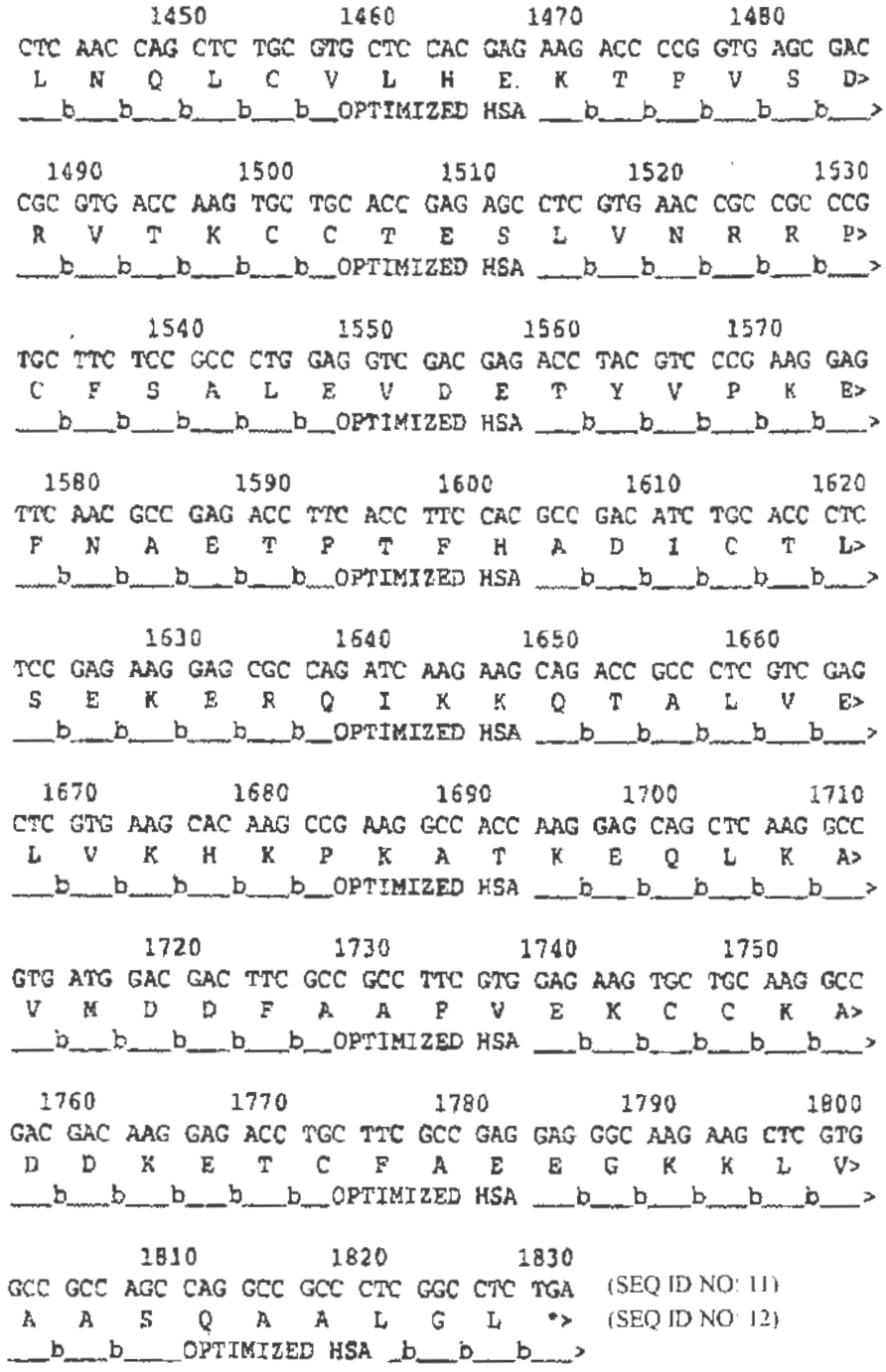
2079 = Total number of nucleotides

457 = Number of nucleotides changed

693 = Total Number of codons

413 = Number of codons changed

FIG 5D


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R V T K C C T E S L V N R R P>
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C F S A L E V D E T Y V P K E>
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F N A E T F T F H A D I C T L>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
1630 1640 1650 1660
TCC GAG AAG GAG CGC CAG ATC AAG AAG CAG ACC GCC CTC GTC GAG
S E K E R Q I K K Q T A L V E>
___b___b___b___b___b__OPTIMIZED HSA ___b___b___b___b___b___>
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Electronic Patent Application Fee Transmittal					
Application Number:	15188478				
Filing Date:	21-Jun-2016				
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS				
First Named Inventor/Applicant Name:	Steven Clyde Perrit				
Filer:	Crissa A. Cook/Brandy Goodman				
Attorney Docket Number:	50091-CNT1				
Filed as Small Entity					
Filing Fees for Utility under 35 USC 111(a)					
Description		**Fee Code**	**Quantity**	**Amount**	**Sub-Total in USD($)**
Basic Filing:					
Pages:					
Claims:					
Miscellaneous-Filing:					
Petition:					
Patent-Appeals-and-Interference:					
Post-Allowance-and-Post-Issuance:					
Extension-of-Time:					

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
			Total in USD ($)	**300**

Electronic Acknowledgement Receipt	
EFS ID:	35453508
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	18-MAR-2019
Filing Date:	21-JUN-2016
Time Stamp:	16:22:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$300
RAM confirmation Number	031919INTEFSW00003543190522
Deposit Account	190522
Authorized User	Brandy Goodman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		PTO_Amendment_18Mar19_50091-CNT_1268730.PDF	135044	yes	9

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Arguments/Remarks Made in an Amendment	6	9
Claims	4	5
Specification	3	3
Drawings-only black and white line drawings	2	2
Amendment/Req. Reconsideration After Non-Final Reject	1	1

Warnings:

Information:

2	Sequence Listing (Text File)	PTO_Sequence_Listing_18Mar19_50091-CNT_1268411.TXT	44420	no	-

Warnings:

Information:

3	Transmittal Letter	PTO_Sequence_Statement_18Mar19_50091-CNT_1268728.PDF	79166	no	1

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Information:

4	Extension of Time	PTO_EOT_18Mar19_50091-CNT_1266711.PDF	164386	no	2

Warnings:

Information:

5	Drawings-only black and white line drawings	PTO_Replacement_Sheets_18 Mar19_50091-CNT_1267680. PDF	680322 [illegible]	no	2
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6	Fee Worksheet (SB06)	fee-info.pdf	30726 [illegible]	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	1134064		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Remarks:

Claims 35-47 remain for consideration in this application, with claim 35 being in independent format. The claims have been amended to specify that the supplement or complete media is a cell culture media supplement or complete media composition for improving cell growth in cell culture. Claim 35 has been further amended to specify that the albumin is mammalian albumin, as described on page 51, line 10. Further, claim 35 has been amended to specify that the recombinant albumin is produced in a transgenic plant. New claims have been added to further specify human serum albumin, and transgenic grain and/or rice, as described and exemplified throughout the application. Entrance of these amendments and favorable consideration of the following remarks is respectfully requested.

Specification and Drawings

A substitute Sequence Listing text file is submitted herewith to include the sequences previously listed in Figs. 1(A)-(D) and Figs. 5(A)-(D). The drawings have likewise been amended to include sequence identifiers at the end of each sequence in Fig. 1(D) and Fig. 5(D), respectively.

The specification has been amended herein to update the Related Applications section to refer to the abandoned status of the parent case The specification has also been amended to include a paragraph referencing the Sequence Listing text file.

The amendments add no new matter.

It is believed that the objections have been traversed.

35 U.S.C. 112, second paragraph

Claims 35-44 have been rejected as being indefinite. The claims have been amended to adjust the claim format and it is believed to address the Examiner's concerns. The claims now clarify that they are directed to compositions for improving cell growth in cell culture as a supplement or complete media formulation. Applicant respectfully submits that the term "improve" is not intended to convey any particular degree of improvement, and in the context of cell culture media and media supplements the term "improve" would be clear and understood.

Claims 43-44 have been rejected for being unclear in terms of the ranges. Applicant has revised the recited ratios so that it is believed that the scope of the ranges is now clear.

Should the Examiner have further recommendations for improving the claim terminology, he is encouraged to contact the undersigned.

35 U.S.C. 101

Claims 35-44 have been rejected as being directed to a product of nature. Reconsideration is respectfully requested. As noted, the claims are directed to a cell culture supplement or complete media composition. The composition comprises a recombinant mammalian albumin that has been produced in a transgenic plant. Further, the albumin has less than 1 EU of endotoxin / mg of albumin, and less than 2% aggregated albumin. Applicant respectfully submits that mammalian albumin produced in a transgenic rice is not a naturally occurring product. Further, albumin that has less than 1 EU of endotoxin / mg of albumin, and less than 2% aggregated albumin is also not naturally occurring.

Applicant respectfully submits that those skilled in the art would immediately recognize that the recombinant albumin produced in a transgenic plant will be different from a naturally occurring mammalian albumin. For example, it will have a different fatty acid profile, and other molecules will attach to the albumin, including heterologous proteins from the host expression system that would not be found in the naturally occurring albumin and would have a different effect in the cell culture medium. Further, the level of glycation of the expressed protein is widely recognized to differ in plant expression systems. Applicant notes, this is exemplified by the data in the present application, such as Fig. 10 (Example 7). CellPrime is a commercial product comparison tested with the inventive albumin compositions. CellPrime is expressed in yeast. SeraCare is a bovine serum albumin. As can be seen, and discussed in Example 7, the different albumins had differential effects on the cultured cells.

Further, the low endotoxin and aggregation values in the claimed composition are also not naturally occurring. Albumin naturally binds endotoxin, which is found in Gram-negative bacteria. Gram-negative bacteria are found everywhere, in virtually all environments on Earth that support life. Thus, endotoxin is likewise found virtually everywhere. When albumin is extracted, such as from a seed, it will be exposed to and bind endotoxin. In the claimed compositions, the albumin has an endotoxin and aggregation level that is not naturally found. It is achieved through a purification process, as described in detail on page 98 of the application involving the use of a detergent to release the endotoxin, followed by removing the detergent via a resin to yield albumin suitable for cell culture media. Accordingly, it is clear that the claimed compositions are not products of nature or naturally occurring under 35 U.S.C. 101, and withdrawal of the rejection is requested.

35 U.S.C. 102

Claims 35-40 have been rejected as being anticipated by Mawal et al. (1987) in view of Davies (US 5,917,022).

As discussed above, the claims have been clarified to be directed to a recombinant mammalian albumin produced in a transgenic plant. Accordingly, this rejection based upon the rice seed albumin of Mawal et al. is traversed.

Claims 35-40 have been rejected as being anticipated by Deeter et al. (WO2007/002762) as evidenced by Davies. In the Action, it was asserted that the composition in Deeter et al. "would inherently lack any endotoxin." Reconsideration and withdrawal is requested. As noted above, endotoxin is not "only found" in the cell wall of Gram-negative bacteria Rather, it is found anywhere that Gram-negative bacteria are found, and can be bound by albumin. However, this was not appreciated by prior art approaches for generating albumin for cell culture media. This is why these references are silent as to endotoxin levels. Based upon Applicant's experience, the prior art processes were likely at levels of about 10 EU and up to 20 EU of endotoxin / mg of albumin. In view of the ubiquitous nature of endotoxin and the ability of albumin to bind endotoxin, further processing techniques had to be developed and applied to the expressed protein to obtain the low endotoxin levels recited. As noted above, the process involves a detergent to release bound endotoxin, followed by a resin to remove the detergent. This is not taught or suggested in Deeter and accordingly, Deeter would not have inherently achieved this low level of endotoxin based upon the information conveyed.

Claim 35 has been rejected as being anticipated by Berezenko et al. (US 6,034,221). The Action asserts that Berezenko does not teach that its albumin is produced in a plant, but argues that this is a product-by-process limitation that does not limit the product itself Again, reconsideration is requested. In fact, the process and expression system *does* yield a different product, with different characteristics. Applicant notes that Berezenko is a Novozymes patent and relates to the CellPrime product that is compared in the patent application. It is expressed in a yeast expression system. As shown in Fig. 10, it has a significantly different impact on cell growth when used in cell culture. As discussed in Example 7, the claimed albumin produced in a transgenic plant with the low endotoxin was 5 times more active in promoting cell viability than the CellPrime product. This data provides the objective evidence requested in the Action by the Examiner to show that the process of expression in a transgenic plant does yield a product that is "markedly different and unexpected" from the prior art of record. Accordingly, reconsideration and withdrawal of this

rejection is requested. Further, as noted above, there is no basis for assuming that the low endotoxin levels would inherently be present in Berezenko.

35 U.S.C. 103

Claims 41-44 have been rejected as being obvious over Deeter et al. in view of Davies, Luyten et al., and Potter et al. Applicant respectfully submits that this rejection is moot in view of the discussion above. Specifically, neither Luyten nor Potter overcome the deficiencies of Deeter et al. noted above. Accordingly, reconsideration and withdrawal of this rejection is also requested.

Conclusion

In view of the foregoing, a Notice of Allowance appears to be in order and such is courteously requested. **Should any additional issues remain that could be facilitated by a telephone interview, the Examiner is encouraged to contact the undersigned.** Any fee which is due in connection with this response should be applied against Deposit Account No. 19-0522

Respectfully submitted,

/Crissa A. Cook/

By _____

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HOVEY WILLIAMS LLP
10801 Mastin Boulevard, Suite 1000
Overland Park, KS 66210
913-647-9050

ATTORNEYS FOR APPLICANT(S)

Claim Amendments:

1.-34. (Canceled).

35. (Currently Amended) A cell culture media supplement or complete media composition~~medium~~ for improving the growth of a cell in cell culture comprising:

a recombinant mammalian albumin wherein said albumin is:

i) produced in a transgenic plant;

ii) has less than 1 EU of endotoxin / mg of albumin; and

iii) less than 2% aggregated albumin~~; and~~

~~wherein said supplement or complete medium improves growth of a cell in cell culture medium comprising said supplement or complete medium~~.

36. (Currently Amended) The ~~supplement~~composition of claim 35, wherein said recombinant albumin comprises at least 0.01 % wt / wt of a heat shock protein.

37. (Currently Amended) The ~~supplement~~composition of claim 36, wherein said heat shock protein is a rice heat shock protein.

38. (Currently Amended) The ~~supplement~~composition of claim 36, wherein said heat shock protein is selected from said group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein.

39. (Currently Amended) The ~~supplement~~composition of claim 36, wherein said heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1), EEC69073 / OsI_37938, and AAB63469.

40. (Currently Amended) The ~~supplement~~composition of claim 36, wherein said supplement comprises at least 0.04 % wt / wt HSP70.

41. (Currently Amended) The ~~supplement~~composition of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is lactoferrin.

42. (Currently Amended) The ~~supplement~~composition of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is transferrin.

43. (Currently Amended) The ~~supplement~~composition of claim 42, wherein said ratio of said transferrin to said recombinant albumin is 1:50 to 1:5000~~1 to 50, to 1 to 5000~~.

44. (Currently Amended) The ~~supplement~~composition of claim 43, wherein said ratio of said lactoferrin to said recombinant albumin is 1:3 to 1:0.33~~1 to 3, to 1 to 0.33~~.

45. (New) The composition of claim 35, wherein said albumin is recombinant human serum albumin.

46. (New) The composition of claim 35, wherein said transgenic plant is a transgenic grain.

47. (New) The composition of claim 46, wherein the transgenic grain is transgenic rice

Amendments to the Specification:

Please replace the Sequence Listing text file with the substitute Sequence Listing text file, submitted herewith.

In the specification on page 1, immediately following the title, please update the "Related Applications" section as follows:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Serial No. 12/798,462, filed February 18, 2010, now abandoned, which claims the benefit of US provisional patent application No. 61,154,204, filed February 20, 2009, the entire contents of which are incorporated herein by reference.

In the specification on page 1, immediately following the "Cross-Reference to Related Applications" section, please add the following paragraph:

SEQUENCE LISTING

The following application contains a sequence listing in computer readable format (CRF), submitted as a text file in ASCII format entitled "SequenceListing," created on March 18, 2019, as 44 KB. The content of the CRF is hereby incorporated by reference.

Amendments to the Drawings:

Please replace the originally-submitted sheets 4 and 11 with the Replacement Sheets, labeled as such, containing updated Fig. 1D and Fig. 5D with SEQ ID NOs.

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15188478 Document Date: 03/18/2019

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

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Form Revision Date: August 26, 2013

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/188,478	Filing Date 06/21/2016	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $210 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	03/18/2019	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
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	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
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	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write '0' in column 3.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/APRIL L. WALKER/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Ser Thr Arg Ile Pro Lys Val Gln Gln Leu Leu Arg Asp Tyr Phe Glu
370 375 380

Gly Lys Glu Pro Asn Lys Gly Val Asn Pro Asp Glu Ala Val Ala Tyr
385 390 395 400

Gly Ala Ala Val Gln Gly Ser Ile Leu Ser Gly Glu Gly Gly Asp Glu
405 410 415

Thr Lys Asp Ile Leu Leu Leu Asp Val Ala Pro Leu Thr Leu Gly Ile
420 425 430

Glu Thr Val Gly Gly Val Met Thr Lys Leu Ile Pro Arg Asn Thr Val
435 440 445

Ile Pro Thr Lys Lys Ser Gln Val Phe Thr Thr Tyr Gln Asp Gln Gln
450 455 460

Thr Thr Val Ser Ile Gln Val Phe Glu Gly Glu Arg Ser Met Thr Lys
465 470 475 480

Asp Cys Arg Leu Leu Gly Lys Phe Asp Leu Ser Gly Ile Pro Ala Ala
485 490 495

Pro Arg Gly Thr Pro Gln Ile Glu Val Thr Phe Glu Val Asp Ala Asn
500 505 510

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APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

23599 7590 06/17/2019

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER
ROLLAND, PAUL F

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
06/17/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

uspatents@hoveywilliams.com

Office Action Summary	Application No. 15/188,478	Applicant(s) Pettit et al.	
	Examiner PAUL J HOLLAND	Art Unit 1656	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.

- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.

Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 03/18/2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 35-47 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 35-47 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____

4) ☐ Other: _____

DETAILED CORRESPONDENCE

Application Status

1. The present application is being examined under the pre-AIA first to invent provisions.

2. Applicant's amendment to the claims filed on 03/18/2019 in response to the Non-Final Rejection mailed on 10/16/2018 is acknowledged. This listing of claims replaces all prior listings of claims in the application.

3. New claims 45-47 are added.

4. Claims 35-47 are pending.

5. Applicant's remarks filed on 03/18/2019 in response to the Non-Final Rejection mailed on 10/16/2018 have been fully considered and are deemed persuasive to overcome at least one of the rejections and/or objections as previously applied.

The text of those sections of Title 35 U.S. Code not included in the instant action can be found in the prior Office Action.

Specification/Drawings

6. The objection to the specification and drawings under 37 CFR 1.821(a)(1) and (a)(2) is withdrawn in view of applicant's amendment to the specification to incorporate sequence identifiers to the sequences in Figures 1 and 5 and filing of a computer readable form (CRF) copy of the "Sequence Listing", an initial paper copy of the "Sequence Listing", and an amendment directing its entry into the specification.

7. The objection to the disclosure is withdrawn in view of applicant's amendment to the specification on 03/18/2019 to indicate the abandonment of the '462 application.

Claim Rejections - 35 USC § 112(b), or Second Paragraph

8. The rejection of claims 35-44 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, for the relative term "improves" is withdrawn in view of applicant's amendment to the claims to remove said term.

9. The rejection of claims 43-44 under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, for reciting a broad range or limitation together with a narrow range or limitation is withdrawn in view of applicant's amendment to the claims.

Claim Rejections - 35 USC § 101

10. The rejection of claims 35-44 under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception is withdrawn in view of applicant's remarks on p. 7 which point to Example 7 in the specification that the mammalian albumin produced in a transgenic plant has differential effects on the cultured cells in comparison to other expression systems, which the disclosure attributes to heat shock proteins found in the plants. Accordingly, the structure of the recombinant mammalian albumin is markedly different than a naturally occurring mammalian albumin.

Claim Rejections - 35 USC § 102

11. The rejection of claims 35-40 under pre-AIA 35 U.S.C. 102(b) as being anticipated by Mawal et al. (*Bioscience Reports*, 1987; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018) is withdrawn in view of applicant's amendment to the claims to recite "recombinant mammalian albumin".

12. The rejection of claim 35 under pre-AIA 35 U.S.C. 102(b) as being anticipated by Berezenko et al. (**US Patent** 6,034,221, 2000; cited on PTO-892 mailed 10/16/2018) is

withdrawn in view of applicant's remarks on p. 8 which demonstrate that the albumin of Berenzenko et al. is similar to the CelPrime product that is compared in the present application, wherein the albumin produced in a transgenic plant as opposed to yeast is 5 times more active in promoting cell viability, which supports that the products are distinct.

13. The rejection of claims 35-40 under pre-AIA 35 U.S.C. 102(b) as being anticipated by Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018) is maintained for the reasons of record and the reasons set forth below. The rejection has been modified in order to incorporate new claims 45-47.

Claims 35-40 and 45-47 are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018).

14. With respect to claims 35-40 and 45-47, Deeter et al. teach components of cell culture medium produced from plant cells that improve the growth of a cell in the cell culture medium wherein the medium comprises human serum albumin produced in rice [see Abstract; paragraphs 0009 and 0116; Example 7]. Deeter et al. also teach clarifying the extracted rice human serum albumin by filtration and purification to approximately 98% (interpreted as no aggregation) [see paragraph 0116]. Although Deeter et al. does not explicitly teach that the supplement comprises less than about 1 EU of Endotoxin/mg of albumin, the reference of Davies is cited in accordance with MPEP 2131.01.II to demonstrate that endotoxin is only found as lipopolysaccharides in the cell wall of Gram-negative bacteria [see Column 1, lines 17-48].

Since the recombinant human serum albumin is produced in rice and is not a bacteria, then the human serum albumin taught by Deeter et al. would inherently lack any endotoxin.

Regarding claims 36-40, Deeter et al. is silent as to the presence of heat-shock proteins, for example those recited in claims 39 and 40, in the albumin preparation. Deeter's method of preparing recombinant albumin from rice grain [see Example 3 at paragraphs 103-105; Example 7 at 116-117), however, is identical to the one employed by applicants [see Example 3 and Example 5]. It is the examiner's position that absent evidence otherwise, these proteins would be inherent to the recombinant human serum albumin produced by Deeter et al. MPEP 2112.I states "[T]he discovery of a previously unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new to the discoverer." *Atlas Powder Co. v. Ireco Inc.,* 190 F.3d 1342, 1347, 51 USPQ2d 1943, 1947 (Fed. Cir. 1999)". Since the Office does not have the facilities for examining and comparing applicants' protein with the protein of the prior art, the burden is on the applicant to show a novel or unobvious difference between the claimed product and the product of the prior art (i.e., that the rice albumin of the prior art does not possess the same material structural and functional characteristics of the claimed supplement comprising recombinant albumin). See *In re Best*, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and *In re Fitzgerald* et al., 205 USPQ 594.

Claim Rejections - 35 USC § 103

15. The rejection of claims 41-44 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018) in view of

Luyten et al. (**US Patent** 6,617,161, 2003; cited on PTO-892 mailed 10/16/2018) and Potter et al. (**US Patent** 5,985,289, 1999; cited on PTO-892 mailed 10/16/2018) is maintained for the reasons of record and the reasons set forth below. The rejection has been modified in order to address applicant's amendment to the claims to incorporate new claims 45-47.

16. The relevant teachings of Deeter et al. as applied to claims 35-40 and 45-47 are set forth above.

With respect to claims 41-44, Deeter et al. teach components of cell culture medium produced from plant cells that improve the growth of a cell in the cell culture medium wherein the medium comprises the addition of albumin, lactotransferrin, or transferrin recombinantly produced from plants [see Abstract; paragraphs 0006, 0009, 0061, 0071, and 0116; Example 7] at concentrations of 0.01-50 mg/mL of lactoferrin [see Example 5; paragraph 0111].

However, Deeter et al. does not teach the supplement of claim 41 comprising both albumin and lactoferrin; the supplement of claim 42 comprising both albumin and transferrin; the supplement of claim 43, wherein said ratio of said transferrin to said recombinant albumin is 1:50 to 1:5000; and the supplement of claim 44, wherein said ratio of said lactoferrin to said recombinant albumin is 1:3 to 1:0.33.

Luyten et al. teach a complete culture medium for mammalian cells comprising 6.25μg (0.00625mg) transferrin and 1.25mg bovine serum albumin per mL, i.e. a ratio of 1 to 200 [see Column 3, lines 16-62; Table 1; see also Example 1, using the medium]. Regarding the full scope of ratios, Luyten et al. teach that the concentrations of the components can be adjusted to suit cell lines as necessary. [Column 3, lines 64-67].

Potter et al. teach that transferrin and lactoferrin are both iron-chelating proteins [see Column 7, lines 15-16]. Lactoferrin is therefore a "transferrin-related protein." Potter et al. also teach that iron-chelating proteins can be employed in culture media at varying concentrations to modulate iron content in the media as necessary for the cells [see Column 6, line 66, through column 7, line 21].

At the time the invention was made it would have been obvious to one of ordinary skill in the art to combine the teachings of Deeter et al., Luyten et al., and Potter et al. according to the teachings of Luyten et al. and Potter et al. because Deeter et al. teach that albumin, transferrin, or lactoferrin produced in plants can be used to improve the growth of cells in a culture medium. Luyten et al. teach that the albumin and transferrin can be combined in a culture medium for cell growth. Potter et al. teach that lactotransferrin or transferrin can be employed in culture media at varying concentrations to modulate iron content in the media necessary for the cells. An ordinarily skilled artisan would have had a reasonable expectation of success, a reasonable level of predictability, and would have been motivated to combine the teachings of Deeter et al., Luyten et al., and Potter et al. because Luyten et al. acknowledges that albumin and transferrin can be combined in cell culture growth medium and Potter et al. acknowledges that both lactotransferrin and transferrin can be added to culture medium to modulate iron content. Therefore, the above invention would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made.

Furthermore, MPEP 2144.06.I states "It is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of

combining them flows logically from their having been individually taught in the prior art." *In re Kerkhoven*, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980) (citations omitted) (Claims to a process of preparing a spray-dried detergent by mixing together two conventional spray-dried detergents were held to be *prima facie* obvious.). See also *In re Crockett*, 279 F.2d 274, 126 USPQ 186 (CCPA 1960) (Claims directed to a method and material for treating cast iron using a mixture comprising calcium carbide and magnesium oxide were held unpatentable over prior art disclosures that the aforementioned components individually promote the formation of a nodular structure in cast iron.); and *Ex parte Quadranti*, 25 USPQ2d 1071 (Bd. Pat. App. & Inter. 1992) (mixture of two known herbicides held *prima facie* obvious)." In the instant case, the improvement in cell growth by addition of plant derived albumin, lactotransferrin, or transferrin was known in the prior art as taught by Deeter et al. Therefore, it would have been obvious to one of ordinary skill in the art to combine these proteins as part of a culture medium to improve cell growth.

Although the combination of Deeter et al., Luyten et al., and Potter et al. do not explicitly teach the ratios of lactoferrin of 1 to 3, to 1 to 0.33, MPEP 2144.05.II.A states, "Generally, differences in concentration or temperature will not support the patentability of subject matter encompassed by the prior art unless there is evidence indicating such concentration or temperature is critical. "[W]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." *In re Aller,* 220 F.2d 454, 456, 105 USPQ 233, 235 (CCPA 1955)". In the instant case, it would be obvious to one of ordinary skill in the art to determine the

appropriate workable ranges based on the teachings of Deeter et al., Luyten et al., and Potter et al.

Response to Remarks Regarding Prior Art Rejections

17. Beginning on p. 8 of applicant's remarks, applicants contend that the prior art processes likely result in levels of endotoxin of about 10 EU and up to 20 EU of endotoxin/mg of albumin due to the ubiquitous nature of endotoxin and that it was not appreciated by prior art approaches.

This argument is found to be not persuasive because MPEP 716.01(c).III states "[a]lthough factual evidence is preferable to opinion testimony, such testimony is entitled to consideration and some weight so long as the opinion is not on the ultimate legal conclusion at issue. While an opinion as to a legal conclusion is not entitled to any weight, the underlying basis for the opinion may be persuasive. *In re Chilowsky,* 306 F.2d 908, 134 USPQ 515 (CCPA 1962)...[a]lthough an affidavit or declaration which states only conclusions may have some probative value, such an affidavit or declaration may have little weight when considered in light of all the evidence of record in the application. *In re Brandstadter,* 484 F.2d 1395, 179 USPQ 286 (CCPA 1973)". In the instant case, given that Deeter et al. uses an identical expression system, it is the examiner's position that the endotoxin level would be inherent to the Deeter et al. composition. Indeed, applicant's disclosure in Example 7 states that "[g]iven the possibility that other commercially available albumin products may have similar overall purity, endotoxin, and detergent levels to rice derived recombinant albumin...". There is no evidence of record to support that the albumin of Deeter et al. does not possess the same characteristics of the claimed compositions. Since the Office does not have the facilities for examining and

comparing applicants' protein with the protein of the prior art, the burden is on the applicant to show a novel or unobvious difference between the claimed product and the product of the prior art (i.e., that the protein of the prior art does not possess the same material structural and functional characteristics of the claimed protein). See *In re Best*, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and *In re Fitzgerald* et al., 205 USPQ 594.

After Final Consideration Program 2.0

18. Applicant is advised that an After-Final Consideration Program [AFCP 2.0] is now available for the consideration of an after-final amendment to the claims that does not broaden the scope of any claim. Applicant is hereby notified that they can participate in this program by filing an AFCP 2.0 Request Form, PTO/SB/434, the required fee, the further amendment, and a statement that the Applicant is willing and available to participate in any interview requested by the examiner concerning the submission. More information on this and other new initiatives at the Patent Office can be found at http://www.uspto.gov/patents/init_events/patapp-initiatives-timeline.jsp or by calling the examiner or his/her supervisor, Manjunath Rao.

Conclusion

19. Status of the claims:

Claims 35-47 are pending.

Claims 35-47 are rejected.

No claims are in condition for an allowance.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to PAUL J HOLLAND whose telephone number is (571)270-3537. The examiner can normally be reached on Monday to Friday from 8AM to 5PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR

system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST text search (see search notes)	10/11/2018	P.H.
Reviewed search history of 12/708462	10/11/2018	P.H.
Inventor and Assignee search (PALM and EAST)	10/11/2018	P.H.
Updated Inventor and Assignee search (PALM and EAST)	06/11/2019	P.H.

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2019/06/11 12:34
L2	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2019/06/11 12:34
L3	131	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2019/06/11 12:34
L4	2	L3 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/06/11 12:34
L5	25	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2019/06/11 12:35
L6	4	L5 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/06/11 12:35
S1	2	"6617161".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:09
S2	2	"6034221".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:10
S3	2	"5986289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:11
S4	2	"5917022".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:14

EAST Search History (Prior Art)

S5	2	"6733746".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:52
S6	2	"5985289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/21 16:34
S7	11	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S8	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S9	106	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S10	2	S9 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:47
S11	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:48
S12	4	S11 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:48
S13	12	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S14	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S15	107	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S16	2	S15 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:39
S17	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39

EAST Search History (Prior Art)

S18	4	S17 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:40
S19	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S20	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S21	126	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S22	2	S21 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:23
S23	25	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:24
S24	4	S23 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:24

EAST Search History (Interference)

		<This search history is empty>			

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	15188478	Filing Date	2016-06-21	Docket Number (if applicable)	50091-CNT	Art Unit	1656
First Named Inventor	Steven Clyde Pettit			Examiner Name	Paul J. Holland		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C. 371, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV.

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

☐ Other _____

☒ Enclosed

☒ Amendment/Reply

☐ Information Disclosure Statement (IDS)

☒ Affidavit(s)/ Declaration(s)

☒ Other Two-Month Extension of Time

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 190522

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
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Signature of Registered U.S. Patent Practitioner			
Signature	/Crissa A. Cook/	Date (YYYY-MM-DD)	2019-11-18
Name	Crissa A. Cook	Registration Number	64054

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Remarks:

Claims 35-47 remain for consideration in this application, with claim 35 being in independent format. Applicant notes, with appreciation, that many of the issues raised in the previous Office Action have been withdrawn.

Turning to the remaining issues, claims 35-40 and 45-47 have been rejected as being anticipated by Deeter et al. (WO 2007/002762) as evidenced by Davies (US 5,917,022). Claims 41-44 have been rejected as being obvious over Deeter et al. (as evidenced by Davies) in view of Luyten et al. (US 6,617,161) and Potter et al. (US 5,985,289). The Office Action acknowledges that "Deeter et al. does not explicitly teach that the supplement comprises less than about 1 EU of Endotoxin/mg of albumin." Page 4. However, the Action asserts that "[s]ince the recombinant human serum albumin is produced in rice and is not a bacteria, then the human serum albumin taught by Deeter et al. would inherently lack any endotoxin." Page 5. The Action relies on Davies as teaching that endotoxin is "only" found in the cell wall of Gram-negative bacteria. Reconsideration and withdrawal of these rejections is requested.

The Examiner's attention is directed to a technical declaration submitted herewith by Dr. Lorne Jordan. The declaration contains data and an explanation of a direct comparison of the albumin produced in Deeter et al., and the albumin produced in the current application. Dr. Jordan first explains that although endotoxin is, indeed, associated with Gram-negative bacteria, these are ubiquitous organisms. Under standard extraction conditions, endotoxin is a common contaminant in protein produced in recombinant systems. Further, it has an unusually strong interaction with albumin, such that *any* endotoxin contamination in the process will be bound by albumin yielding uniquely higher contamination levels in recombinant albumin. Dr Jordan explains that although it was not reported in Deeter et al., endotoxin *was* present in the albumin produced at that time. Techniques had to be researched and developed to reduced/remove endotoxin contamination The result was the unexpected ability to achieve ultra-low[1] levels of endotoxin contamination now claimed.

Dr. Jordan also notes that the current application itself actually contains a comparison of the Deeter et al.-produced albumin and the albumin in the current application. In fact, Example 5 of the current application is identical to the protocol described in Example 7 of Deeter et al The current application designates this protocol as process "B000." Further, Table E3 in the current application shows the endotoxin levels detected in albumin extracted and purified using the old

[1] For reference, the initial standard was to achieve less than 10 EU/mg.

"B000" Deeter et al. process. One batch was able to achieve 4.4 EU/mg endotoxin, while others were above 90 EU/mg endotoxin. The average endotoxin level in the Deeter et al.-produced albumin was **44.97 EU/mg**. This data objectively demonstrates that the albumin produced in Deeter et al. contained significant levels of endotoxin. Thus, the premise of the rejection (that Deeter et al. inherently lacked any endotoxin) is not correct.

Further, in the context of using the albumin for cell culture, Table E3 demonstrates that there was a detrimental effect on cell viability as the level of protein was increased for the Deeter et al.-produced albumin. In contrast, Dr. Jordon notes that the new protocol, "B0000C," achieved significantly lower endotoxin levels, well under 1 EU/mg, with an average of only **0.36 EU/mg**. This is demonstrated by the data presented in Table E4. Applicant notes that these tables were presented on pages 97-98 of the application as filed. Moreover, there is no significant variation on cell viability when the inventive albumin is used in cell culture at various concentrations.

In summary, comparing data from the two protocols, B000 (Deeter et al.) and B0000C (current invention), clearly demonstrates that the endotoxin levels in Deeter et al. were well above the recited "less than 1 EU of endotoxin / mg of albumin." Therefore, Deeter et al. cannot be considered to anticipate the claims, as it does not "inherently" contain "less than 1 EU of endotoxin / mg of albumin." Further, Applicant respectfully submits that the secondary references do not remedy the deficiencies of Deeter et al. Accordingly, even if Deeter et al. is combined with the secondary references, one of ordinary skill in the art still fails to arrive at the claimed invention. Therefore, it is respectfully requested that the remaining rejections be withdrawn.

In view of the foregoing, a Notice of Allowance appears to be in order and such is courteously requested. **Should any additional issues remain that could be facilitated by a telephone interview, the Examiner is encouraged to contact the undersigned.** Any fee which is due in connection with this response should be applied against Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Cook/

By _____

Crissa A. Cook, Reg. No. 64,054
HOVEY WILLIAMS LLP
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Overland Park, KS 66210
913-647-9050

ATTORNEYS FOR APPLICANT(S)

Claim Amendments:

1.-34. (Canceled).

35. (Previously Presented) A cell culture media supplement or complete media composition for improving the growth of a cell in cell culture comprising:

a recombinant mammalian albumin wherein said albumin is:

i) produced in a transgenic plant;

ii) has less than 1 EU of endotoxin / mg of albumin; and

iii) less than 2% aggregated albumin

36. (Previously Presented) The composition of claim 35, wherein said recombinant albumin comprises at least 0.01 % wt / wt of a heat shock protein.

37. (Previously Presented) The composition of claim 36. wherein said heat shock protein is a rice heat shock protein.

38. (Previously Presented) The composition of claim 36, wherein said heat shock protein is selected from said group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein

39. (Previously Presented) The composition of claim 36, wherein said heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI 37938, and AAB63469.

40. (Previously Presented) The composition of claim 36, wherein said supplement comprises at least 0.04 % wt / wt HSP70.

41. (Previously Presented) The composition of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is lactoferrin.

42. (Previously Presented) The composition of claim 36, further comprising a recombinant transferrin related protein, wherein said transferrin related protein is transferrin.

43. (Previously Presented) The composition of claim 42, wherein said ratio of said transferrin to said recombinant albumin is 1:50 to 1:5000.

44. (Previously Presented) The composition of claim 43, wherein said ratio of said lactoferrin to said recombinant albumin is 1:3 to 1:0.33.

45. (Previously Presented) The composition of claim 35, wherein said albumin is recombinant human serum albumin.

46. (Previously Presented) The composition of claim 35, wherein said transgenic plant is a transgenic grain.

47. (Previously Presented) The composition of claim 46, wherein the transgenic grain is transgenic rice.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Group Art Unit No.: 1656
Filed: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. HOLLAND

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDMENT

In response to the Office Action dated June 17, 2019, amendment and reconsideration of the above-referenced application is requested.

Claim Amendments begin on page 2.

Remarks begin on page 4.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Group Art Unit No.: 1656
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CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. HOLLAND

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION UNDER 37 C.F.R. 1.132

I, Lorne Jordan, attest and declare as follows.

1. I have a Ph.D. in Biochemistry and Molecular Biophysics from Kansas State University, a Master of Science degree from the University of Oklahoma in Chemistry and Biochemistry, and an undergraduate degree in Biology and Chemistry from Bowling Green State University. I am currently a Scientist, Analytical Biochemistry, at Ventria Bioscience, the assignee of the above-referenced application.

2. I understand that the Examiner has cited WO 2007/002762 (Deeter et al.) against the above-referenced application. I have been asked to provide this declaration to explain the information described in this reference as understood by those skilled in this field.

3. The basic premise asserted by the Examiner on page 5 of the Action is that "the human serum albumin taught by Deeter et al. would inherently lack any endotoxin." This statement is incorrect. In fact, in the process carried out by Deeter et al. endotoxin levels were observed in the final product. However, these values were not reported in the patent application at that time. As noted by the Examiner endotoxin is found in Gram-negative bacteria which are ubiquitous organisms. However, it is not correct to assume that because the Deeter et al. system is plant-based, it would be free of endotoxin contamination. When albumin is extracted from recombinant systems, it is exposed to and binds endotoxin. Endotoxin is a well-known

contaminant of recombinant protein systems. Due to its strong amphiphilic nature, albumin has an uncharacteristically strong interaction with endotoxin (compared to other plasma proteins). This has resulted in the unusually challenging development of a specific and non-obvious purification strategy for achieving recombinant albumin with low endotoxin levels, which was one of the aims of the current invention.

4. The current application actually contains data directly comparing the "old" approach described in Deeter et al. with the current invention. The Examiner's attention is directed to Example 5 ("Purification and characterization of recombinant serum human albumin (rHSA)) in the current Application, also designated as Process "B000". Notably, the protocol in Example 5 is identical to the protocol described in Example 7 of Deeter et al. (paragraphs [0116]-[0117]). Further, the Examiner's attention is directed to Table E3 on page 104 of the current Application. For ease of reference, the table is copied below. This table shows the endotoxin levels detected in albumin extracted and purified using the old "B000" Deeter et al. process.

Table E3
Cellastim - Old Process B000

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					1mg/ml	2 mg/ml	5 mg / ml	10 /mg ml
2/27/08	B202	70.6	25.3	815.3	15	7.9	4.1	0.9
2/27/08	B203	58.8	23.1	267.7	15.8	12.4	6.5	4.1
2/28/08	B204	59.9	29.2	589.2	16.3	11.1	5.2	2.9
2/28/08	B205	63	64.3	133.4	14	11.6	6.9	3.8
3/3/08	B206	41.1	35.1	1182.2	14.9	2.3	0.0	0.0
3/3/08	B207	94.2	28.7	388	16.5	12.7	7.6	4
3/4/08	B208	24.5	>80	467.2	15.3	11.4	7.1	2.7
3/4/08	B209	66.1	91.8	406.4	16.7	15.2	6.9	4.1
3/4/08	B210	87.3	67.8	76.4	17.5	19.1	15.4	8.5
3/11/08	B217	62.09	4.4	127.3	16.7	18.7	13.2	6.6
Averages			**44.97**	**445.31**	**15.87**	**12.24**	**7.29**	**3.76**

As can be seen, significant levels of endotoxin are present in albumin produced using the approach of Deeter et al. Further, this data demonstrates that the endotoxin levels in Deeter et al. were not inherently low or not present, as asserted in the Office Action. Moreover, in the case of the B000 protocol, cell viability is seen to decrease, dose-dependently, from 16 (0.5 mg/mL) to 4 (10 mg/mL).

5. The ability to achieve recombinant albumin with such low endotoxin levels was unexpected. Conceptually, a specific endotoxin-removal step was suggested and utilized with the goal of using detergent to remove endotoxin from protein. A process was developed to leverage the amphiphilic nature of detergent. Detergent and endotoxin interact with one another in an amphiphilic manner to form a more thermodynamically stable phase. Thus, conceptually, detergent was explored to bind and "extract" endotoxin from protein, despite the strong endotoxin-albumin interaction.

However, although detergent was found to successfully separate the endotoxin from the protein of interest, the detergent itself is a contaminant of the end product with detrimental effects on cells in culture. Therefore, the detergent-endotoxin complex must be subsequently removed from the solution. To accomplish this, we developed a protocol to absorb the detergent-endotoxin complex from the solution onto a hydrophobic detergent-binding resin. As a result of the process, protein without endotoxin flows through the chromatography resin/column and detergent-endotoxin complex is bound to the resin and separated from the protein.

6. Following the above concepts, the initial process B000, was designed (Example 5). However, this approach still resulted in high endotoxin levels (44.97 EU/mg average), and also had high detergent levels (445.31 ppm average), which was detrimental to cell viability in a dose-dependent manner, as noted.

7. Thus, a more advanced process, B0000C, was designed using Tr-X-100 detergent and SDR (Pall) hydrophobic resin, as described in the Application. This new approach unexpectedly resulted in endotoxin reduction by 125-fold and detergent reduction by 129-fold compared to the Deeter et a. process B000. Data for the B0000C process are shown in Table E4, copied below for ease of reference.

Table E4 Cellastim - New Process B0000C								
Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					0.5mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/2/2009	B0032C	209.4	0.35	3.1	13.1	15.7	13.4	11.2
2/2/2009	B0033C	271.4	0.26	7.9	18.4	17.4	15.1	14.3
2/10/2009	B0041C	247.1	0.18	5.4	15.4	17.2	14.1	12.1
4/27/2009	B0118C	617.4	0.11	Less than 1	15.7	15.4	19.2	16.8
5/14/2009	B0138C	610.6	0.48	Less than 1	12.5	15.8	15.1	14.6
6/9/2009	B0158C	598.0	0.13	3.9	13.7	17.1	13.2	11
6/15/2009	B0162C	618.6	0.20	2.0	15.5	17.5	17.2	14.7
7/28/2009	B0196C	507.0	0.36	3.9	17.4	17.3	18.8	14.8
8/26/2009	B0219C	851.4	0.86	8.2	17.9	16.3	16.6	13.7
8/31/2009	B0220C	897.8	0.93	Less than 1	13.5	16	15.5	15.1
9/9/2009	B0227C	929.9	0.13	Less than 1	11.1	14.4	12.5	11.4
Averages			**0.36**	**3.45**	**14.92**	**16.37**	**15.52**	**13.6**

As can be seen, the improved process achieves an ***endotoxin average of 0.36 EU/mg*** and ***detergent levels of 3.45 ppm***. Cell viability was also substantially increased by 3x, even at high protein concentration 10 mg/mL indicating the absence of toxicity. Importantly, there is little variation on viability across protein concentration levels.

8. In summary, a comparison of the performance of the original B000 process (Deeter et al.) and the more advanced B0000C process (Currently invention) across several different batches of albumin demonstrates that the original B000 process produced recombinant albumin with high endotoxin content and high detergent content, whereas the advanced B0000C process produced recombinant albumin with significantly lower endotoxin content (less than 1 EU/mg) and detergent. Moreover, a comparison of the performance of the improved B0000C process with low endotoxin also improved cell culture performance as measured by cell viability per ml, especially at higher levels of albumin inclusion in cell culture media

I hereby declare that all statements made herein of my own knowledge are true and that statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Declaration is directed.

Lorne Jordan, Ph.D.

11-17-19
Date

PTO/AIA/22 (03-13)
Approved for use through 7/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Docket Number (Optional) 50091-CNT
Application Number 15/188,478	Filed June 21, 2016
For CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	
Art Unit 1656	Examiner Paul J. Holland

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	Micro Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☑	Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$ 300
☐	Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☐	Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$
☐	Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☑ Applicant asserts small entity status. See 37 CFR 1.27

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

☐ A check in the amount of the fee is enclosed

☐ Payment by credit card. Form PTO-2038 is attached.

☑ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 190522.

☑ Payment made via EFS-Web.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant.

☑ attorney or agent of record. Registration number 64054

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____

/Crissa A. Cook/ — Signature
November 18, 2019 — Date
Crissa A. Cook — Typed or printed name
913-647-9050 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*

☑ * Total of 1 forms are submitted

This collection of information is required by 37 CFR 1.136(a). The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop PCT, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal					
Application Number:	15188478				
Filing Date:	21-Jun-2016				
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS				
First Named Inventor/Applicant Name:	Steven Clyde Pettit				
Filer:	Crissa A. Cook/Jean Kahrau				
Attorney Docket Number:	50091-CNT1				
Filed as Small Entity					
Filing Fees for Utility under 35 USC 111(a)					
Description		**Fee Code**	**Quantity**	**Amount**	**Sub-Total in USD($)**
Basic Filing:					
Pages:					
Claims:					
Miscellaneous-Filing:					
Petition:					
Patent-Appeals-and-Interference:					
Post-Allowance-and-Post-Issuance:					
Extension-of-Time:					

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
RCE 1ST REQUEST	2801	1	650	650
		Total in USD ($)		**950**

Electronic Acknowledgement Receipt	
EFS ID:	37776664
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Jean Kahrau
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	18-NOV-2019
Filing Date:	21-JUN-2016
Time Stamp:	12:05:02
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$950
RAM confirmation Number	E2019AHC05307399
Deposit Account	190522
Authorized User	Jean Kahrau
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	PTO_RCE_18Nov19_50091-CNT_1356199-1.PDF	1349886	no	3
Warnings:					
Information:					
2		PTO_Amendment_18Nov19_50091-CNT_1405533-1.PDF	97076	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Arguments/Remarks Made in an Amendment	4	5
	Claims	2	3
	Amendment Submitted/Entered with Filing of CPA/RCE	1	1

Warnings:					
Information:					
3	Affidavit-traversing rejectns or objectns rule 132	PTO_132_Declaration_18Nov19_50091-CNT_1405613-1.PDF	450739	no	5
Warnings:					
The page size in the PDF is too large. The pages should be 8.5 x 11 or A4. If this PDF is submitted, the pages will be resized upon entry into the Image File Wrapper and may affect subsequent processing					
Information:					
4	Extension of Time	PTO_EOT_18Nov19_50091-CNT_1405269-1.PDF	164396	no	2
Warnings:					
Information:					

5	Fee Worksheet (SB06)	fee-info.pdf	32528 [illegible]	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2094625		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1 31-2014. OMB 0651-0032
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 15/188,478	Filing Date 06/21/2016	☐ To be Mailed

ENTITY: ☐ LARGE ☑ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $40 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $210 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT	11/18/2019	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 13	Minus	** 20	= 0		x $50 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write '0' in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/MOLIKI I MAY/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

23580 7590 11/27/2019

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER	
HOLLAND, PAUL J	
ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 11/27/2019

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

TITLE OF INVENTION: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEES DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	02/27/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23589 7590 11/27/2019
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

TITLE OF INVENTION: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	02/27/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
HOLLAND, PAUL J	1656	435-404000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

23580 7590 11/27/2019

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER	
HOLLAND, PAUL J	

ART UNIT	PAPER NUMBER
3656	

DATE MAILED: 11/27/2019

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/188,478	Applicant(s) Pettit et al.	
	Examiner PAUL J HOLLAND	Art Unit 1656	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable. PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to RCE filed on 11/18/2019.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 35-47 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

DETAILED CORRESPONDENCE

Application Status

The present application is being examined under the pre-AIA first to invent provisions.

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 11/18/2019 has been entered.

Applicant's amendment to the claims filed on 11/18/2019 in response to the Final Rejection mailed on 06/17/2019 is acknowledged and entered into the record.

Applicant's remarks and Rule 1.132 declaration filed on 11/18/2019 in response to the Final Rejection mailed on 06/17/2019 have been fully considered and are deemed persuasive to overcome the rejections and/or objections as previously applied.

The text of those sections of Title 35 U.S. Code not included in the instant action can be found in the prior Office Action.

Withdrawn Claim Rejections - 35 USC § 102

The rejection of claims 35-40 and 45-47 under pre-AIA 35 U.S.C. 102(b) as being anticipated by Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018) is withdrawn in view of applicant's declaration filed under Rule 1.132 which demonstrates that the compositions of Deeter et al. are incapable of achieving the claimed less than 1 EU of

endotoxin/mg of albumin and that the levels of endotoxins in the culture medium were not appreciated at the time of Deeter's invention.

Withdrawn Claim Rejections - 35 USC § 103

The rejection of claims 41-44 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) as evidenced by Davies (**US Patent Application** 5,917,022; cited on PTO-892 mailed 10/16/2018) in view of Luyten et al. (**US Patent** 6,617,161, 2003; cited on PTO-892 mailed 10/16/2018) and Potter et al. (**US Patent** 5,985,289, 1999; cited on PTO-892 mailed 10/16/2018) is withdrawn for the reasons set forth above regarding Deeter et al.

Examiner's Statement of Reasons for Allowance

The following is an examiner's statement of reasons for allowance. Claims 35-47 are drawn to a cell culture media supplement or complete media composition for improving the growth of a cell in cell culture comprising: a recombinant mammalian albumin wherein said albumin is: i) produced in a transgenic plant; ii) has less than 1 EU of endotoxin/mg of albumin; and iii) less than 2% aggregated albumin. The closest prior art of record is the reference of Deeter et al. (**WO**2007/002762 A2; cited on PTO-892 mailed 10/16/2018) [see prior Office Actions]. However, as stated above, applicant's declaration filed under Rule 1.132 which demonstrates that the compositions of Deeter et al. are incapable of achieving the claimed less than 1 EU of endotoxin/mg of albumin [see Table E3 and Table E4] and that the levels of endotoxins in the culture medium were not appreciated at the time of Deeter's invention is found to be persuasive. Furthermore, Example 7 in the specification demonstrates that the mammalian albumin produced in a transgenic plant has differential effects on the cultured cells

in comparison to other expression systems, which the disclosure attributes to heat shock proteins found in the plants. Accordingly, the structure of the recombinant mammalian albumin is markedly different than a naturally occurring mammalian albumin.

Therefore, the cell culture media supplement of claims 35-47 are allowable over the prior art of record.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee, and to avoid processing delays, should preferably accompany the issue fee. Such admission should be clearly labeled "Comments on Statement of Reasons for Allowance".

Quick Path Information Disclosure Statement

For situations when the applicant needs to file an IDS *after issue fee has been paid*, The United States Patent and Trademark Office (USPTO) has implemented a Quick Path Information Disclosure Statement (QPIDS) program intended to reduce pendency and applicant costs. This pilot program will permit an examiner to consider an IDS after payment of the issue fee without the need to reopen prosecution, effectively obviating the need to pursue a Request for Continued Examination (RCE). Where the examiner determines that no item of information in the IDS necessitates reopening prosecution, the Office will issue a corrected notice of allowability. In addition to reducing pendency, this program will promote efficiency in the examination process. There will be no fee required to use this program, beyond existing fees, e.g., fees for IDS submission. More information on this and other new initiatives at the Patent Office can be found at http://www.uspto.gov/patents/init_events/patapp-initiatives-timeline.jsp or by calling the examiner or his/her supervisor.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to PAUL J HOLLAND whose telephone number is (571)270-3537. The examiner can normally be reached on Monday to Friday from 8AM to 5PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC						
Symbol					Type	Version
C07K	/	14	/	76	F	2013-01-01
C07K	/	14	/	79	I	2013-01-01
C12N	/	5	/	0056	I	2013-01-01
C12N	/	5	/	0043	I	2013-01-01
C12N	/	2500	/	25	A	2013-01-01
C12N	/	2501	/	998	A	2013-01-01
C12N	/	2501	/	105	A	2013-01-01
C12N	/	2500	/	24	A	2013-01-01
C12N	/	2510	/	02	A	2013-01-01

CPC Combination Sets							
Symbol					Type	Set	Ranking	Version
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	22 November 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20191122

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C07K14/76	/	14	/	76
C07K14/79	/	14	/	79
C12N5/00	/	5	/	00
NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	22 November 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20191122

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner)	(Date)	Total Claims Allowed: 13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656 (Primary Examiner)	22 November 2019 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure NONE

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST text search (see search notes)	10/11/2018	P.H.
Reviewed search history of 12/708462	10/11/2018	P.H.
Inventor and Assignee search (PALM and EAST)	10/11/2018	P.H.
Updated Inventor and Assignee search (PALM and EAST)	06/11/2019	P.H.
Updated Inventor and Assignee search (PALM and EAST)	11/22/2019	P.H.

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
EAST text search (see search notes)		11/22/2019	P.H.

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2019/11/22 15:04
L2	1	l1 and endotoxin	US-PGPUB; USPAT	OR	ON	2019/11/22 15:05
L3	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2019/11/22 15:05
L4	134	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2019/11/22 15:05
L5	11	L4 AND (ENDOTOXIN)	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2019/11/22 15:05
S1	2	"6617161".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:09
S2	2	"6034221".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:10
S3	2	"5986289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:11
S4	2	"5917022".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:14
S5	2	"6733746".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:52

EAST Search History (Prior Art)

S6	2	"5985289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/21 16:34
S7	11	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S8	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S9	106	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S10	2	S9 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:47
S11	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:48
S12	4	S11 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:48
S13	12	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S14	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S15	107	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S16	2	S15 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:39
S17	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S18	4	S17 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:40
S19	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S20	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23

EAST Search History (Prior Art)

S21	126	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S22	2	S21 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:23
S23	25	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:24
S24	4	S23 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:24

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L6	35088	ALBUMIN AND (ENDOTOXIN)	US-PGPUB; USPAT	OR	ON	2019/11/22 15:06
L7	1423	ALBUMIN with (ENDOTOXIN)	US-PGPUB; USPAT	OR	ON	2019/11/22 15:06
L8	19	(ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM)) WITH (ENDOTOXIN)	USPAT	OR	ON	2019/11/22 15:06

Bibliographic Data

Application No: 15/188,478

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /PAUL J HOLLAND/ Examiner's Signature | Initials

Title: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
06/21/2016	530	1656	50091-CNT1
RULE			

APPLICANTS

Ventria BioScience Inc., Fort Collins, CO,

INVENTORS

Steven Clyde Pettit Fort Collins, CO, UNITED STATES

Mary Ann Michelle Fernandez Santos Elk Grove, CA, UNITED STATES

Ning Huang Davis, CA, UNITED STATES

CONTINUING DATA

This application is a CON of 12708462 02/18/2010ABN

12708462 has PRO of 61154204 02/20/2009

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

06/30/2016

**** SMALL ENTITY ****

STATE OR COUNTRY

UNITED STATES

ADDRESS

Hovey Williams LLP

10801 Mastin Blvd., Suite 1000

Overland Park, KS 66210

UNITED STATES

FILING FEE RECEIVED

$70

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	15188478	Filing Date	2016-06-21	Docket Number (if applicable)	50091-CNT	Art Unit	1656
First Named Inventor	Steven Clyde Pettit			Examiner Name	Paul J. Holland		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, to any international application that does not comply with the requirements of 35 U.S.C. 371, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV.

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☐ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☐ Other _____

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 190522

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (07-14)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number

Signature of Registered U.S. Patent Practitioner			
Signature	/Crissa A. Cook/	Date (YYYY-MM-DD)	2019-12-19
Name	Crissa A. Cook	Registration Number	64054

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Patent Application Fee Transmittal	
Application Number:	15188478
Filing Date:	21-Jun-2016
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Perrit
Filer:	Crissa A. Cook/Brandy Goodman
Attorney Docket Number:	50091-CNT1
Filed as Small Entity	

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
RCE- 2ND AND SUBSEQUENT REQUEST	2820	1	950	950
		Total in USD ($)		**950**

Electronic Acknowledgement Receipt	
EFS ID:	38088700
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	19-DEC-2019
Filing Date:	21-JUN-2016
Time Stamp:	14:51:57
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$950
RAM confirmation Number	E2019BIE52300006
Deposit Account	190522
Authorized User	Brandy Goodman
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	PTO_IDS_1of2_19Dec19_50091-CNT1_1422369.PDF	1040224	no	13
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_IDS_2of2_19Dec19_50091-CNT1_1422368.PDF	1037868	no	8
Warnings:					
Information:					
3	Transmittal Letter	PTO_IDS_Transmittal_19Dec19_50091-CNT1_1422372.PDF	46017	no	1
Warnings:					
Information:					
4	Request for Continued Examination (RCE)	PTO_RCE_19Dec19_50091-CNT1_1422272.PDF	1349862	no	3
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30318	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	3504289		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	15188478
Filing Date	2016-06-21
First Named Inventor	Steven Clyde Pettit
Art Unit	1656
Examiner Name	Paul J. Holland
Attorney Docket Number	50091-CNT1

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6733746		2004-05-11	Daley	
	2	4075197		1978-02-21	Schuck	
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	26	SARKAR, Rice sHsp genes: genomic organization and expression profiling under stress and development, 2009, BMC Genomics 10 393 1-16	
	27	KAMPINGA, Guidelines for the nomenclature of the human heat shock proteins, 2009, Cell. Stress Chap. 14 105-111	
	28	PETITT, Enhanced growth and productivity of a hybridoma with recombinant human albumin and lactoferrin, 2009, Bioprocess J. 8(1) 50-55	
	29	INVITRIA, Recombinant Human Serum Albumin Expressed in Plants Improves the Productivity and Growth Kinetics of CHO, Press release, March 2009	
	30	MANTEI, et al, 1981, Nature 293 620-625	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15188478
	Filing Date	2016-06-21
	First Named Inventor	Steven Clyde Pettit
	Art Unit	1656
	Examiner Name	Paul J Holland
	Attorney Docket Number	50091-CNT1

	31	HASHIZUME, 1987, Meth. Enzmol. 147 303-314	
	32	HUANG, 2008, In Vitro Cell Dev. Biol. 44 464-471	
	33	INVITRIA, Press release - Sept 2009	
	34	INVITRIA, Press release -Oct 2009	
	35	TANG, 2010, Wound Rep. Reg 18 123-131	
	36	INVITRIA, Poster January 2010	
	37	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated February 19, 2013	
	38	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated September 18, 2013	
	39	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated June 3, 2015	
	40	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated December 21, 2015	

If you wish to add additional non-patent literature document citation information please click the Add button Add

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15188478
	Filing Date	2016-06-21
	First Named Inventor	Steven Clyde Pettit
	Art Unit	1656
	Examiner Name	Paul J Holland
	Attorney Docket Number	50091-CNT1

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15188478
	Filing Date	2016-06-21
	First Named Inventor	Steven Clyde Pettit
	Art Unit	1656
	Examiner Name	Paul J. Holland
	Attorney Docket Number	50091-CNT1

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Crissa A. Cook/	Date (YYYY-MM-DD)	2019-12-19
Name/Print	Crissa A. Cook	Registration Number	64054

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT1
Steven Clyde Pettit	Confirmation No.: 8304
Serial No.: 15/188,478	Group Art Unit No.: 1656
Filed: June 21, 2016	Customer No.: 23589
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. Holland

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

In response to the Notice of Allowance dated, November 27, 2019, a Request for Continued Examination is submitted herewith to permit consideration of an Information Disclosure Statement containing references previously submitted or cited in parent application Serial No. 12/708,462. Through oversight, the Information Disclosure Statement was not previously submitted in the current application. The attached references are being filed to fulfill the duty of candor and good faith toward the Patent and Trademark Office, as required by 37 C.F.R. §1.56.

The references listed on the attached PTO/SB/08A have been previously submitted or cited in parent application Serial No. 12/708,462, and, therefore, in accordance with MPEP 609, are not being provided at this time. Any fee which is due in connection with this Statement should be applied against Deposit Account No. 19-0522.

Respectfully submitted,

By /Crissa A. Cook/
Crissa A. Cook, Reg. No. 64,054
HOVEY WILLIAMS LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210
913.647.9050

ATTORNEYS FOR APPLICANT(S)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

23569 7590 01/08/2020

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER	
HOLLAND, PAUL J	
ART UNIT	PAPER NUMBER
1656	

DATE MAILED: 01/08/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

TITLE OF INVENTION: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEES DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	04/08/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23589 7590 01/08/2020
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

TITLE OF INVENTION: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	$500	$0.00	$0.00	$500	04/08/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
HOLLAND, PAUL J	1656	435-404000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

EXAMINER
HOLLAND, PAUL J

ART UNIT	PAPER NUMBER
1656	

23580 7590 01/08/2020

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

DATE MAILED: 01/08/2020

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/188,478	Applicant(s) Pettit et al.	
	Examiner PAUL J HOLLAND	Art Unit 1656	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable. PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to RCE filed on 12/19/2019.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 35-47 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08). Paper No./Mail Date 12/19/2019.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413). Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☑ Other DETAILED CORRESPONDENCE.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

DETAILED CORRESPONDENCE

Application Status

The present application is being examined under the pre-AIA first to invent provisions.

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after allowance or after an Office action under *Ex Parte Quayle*, 25 USPQ 74, 453 O.G. 213 (Comm'r Pat. 1935). Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, prosecution in this application has been reopened pursuant to 37 CFR 1.114. Applicant's submission filed on 12/19/2019 has been entered.

Information Disclosure Statement

The information disclosure statements (IDS) submitted on 12/19/2019 were filed after the mailing date of the Notice of Allowance on 11/27/2019. The submission is in compliance with the provisions of 37 CFR 1.97. Accordingly, the information disclosure statements are being considered by the examiner.

The IDSs filed on 12/19/2019 have been considered by the examiner and copies of the Form PTO/SB/08 are attached to the office action.

The newly cited references fail to teach or suggest the claimed cell culture media supplement or complete media compositions of claims 35-47. Accordingly, claims 35-47 are allowable for the reasons already of record set forth in the Notice of Allowance mailed on 11/27/2019.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to PAUL J HOLLAND whose telephone number is (571)270-3537. The examiner can normally be reached on Monday to Friday from 8AM to 5PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PAUL J HOLLAND/
Primary Examiner, Art Unit 1656

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC							
Symbol						Type	Version
C07K	/	14	/	76		F	2013-01-01
C07K	/	14	/	79		I	2013-01-01
C12N	/	5	/	0056		I	2013-01-01
C12N	/	5	/	0043		I	2013-01-01
C12N	/	2500	/	25		A	2013-01-01
C12N	/	2501	/	998		A	2013-01-01
C12N	/	2501	/	105		A	2013-01-01
C12N	/	2500	/	24		A	2013-01-01
C12N	/	2510	/	02		A	2013-01-01

CPC Combination Sets								
Symbol					Type	Set	Ranking	Version
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	03 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20200103

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C07K14/76	/	14	/	76
C07K14/79	/	14	/	79
C12N5/00	/	5	/	00
NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	03 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office — Part of Paper No.: 20200103

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al
	Examiner	Art Unit
	PAUL J HOLLAND	1656

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	13	
/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	03 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office Part of Paper No.: 20200103

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	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST text search (see search notes)	10/11/2018	P.H.
Reviewed search history of 12/708462	10/11/2018	P.H.
Inventor and Assignee search (PALM and EAST)	10/11/2018	P.H.
Updated Inventor and Assignee search (PALM and EAST)	06/11/2019	P.H.
Updated Inventor and Assignee search (PALM and EAST)	11/22/2019	P.H.

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/188,478	Pettit et al.
	Examiner	Art Unit
	PAUL J HOLLAND	1656

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
EAST text search (see search notes)		11/22/2019	P.H.
Updated EAST text search (see search notes)		01/03/2020	P.H.

/PAUL J HOLLAND/ Primary Examiner, Art Unit 1656	

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	2	"6617161".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:09
S2	2	"6034221".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:10
S3	2	"5986289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:11
S4	2	"5917022".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:14
S5	2	"6733746".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/19 16:52
S6	2	"5985289".PN.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/21 16:34
S7	11	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S8	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S9	106	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:46
S10	2	S9 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:47

EAST Search History (Prior Art)

S11	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/05/22 08:48
S12	4	S11 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/05/22 08:48
S13	12	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S14	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S15	107	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S16	2	S15 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:39
S17	23	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2015/12/11 14:39
S18	4	S17 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2015/12/11 14:40
S19	13	((Steven) near2 (Pettit)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S20	1	(("Mary Ann") near2 ("Fernandez Santos")).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S21	126	((Ning) near2 (Huang)).INV.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:23
S22	2	S21 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:23
S23	25	(VENTRIA NEAR2 BIOSCIENCE).AS.	US-PGPUB; USPAT	OR	ON	2018/10/11 11:24
S24	4	S23 AND (ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2018/10/11 11:24

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	1774	(ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM))	US-PGPUB; USPAT	OR	ON	2020/01/03 07:52
L2	88	(ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM)) WITH (ENDOTOXIN)	US-PGPUB; USPAT	OR	ON	2020/01/03 07:52
L3	2	((ALBUMIN NEAR5 (CULTURE NEAR2 MEDIUM)) WITH (ENDOTOXIN)).CLM.	US-PGPUB; USPAT	OR	ON	2020/01/03 07:52

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	15188478
Filing Date	2016-06-21
First Named Inventor	Steven Clyde Pettit
Art Unit	1656
Examiner Name	Paul J. Holland
Attorney Docket Number	50091-CNT1

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6617161		2003-09-09	Luyten	
	2	6034221		2000-03-07	Berezenko, et al	
	3	5985289		1999-11-16	Potter, et al	
	4	5917022		1999-06-29	Davies	

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	39	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated June 3, 2015	
	40	Copy of Office Action in corresponding U.S. Patent Application No. 12/708,462, dated December 21, 2015	

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	First Named Inventor	Steven Clyde Pettit
	Art Unit	1656
	Examiner Name	Paul J. Holland
	Attorney Docket Number	50091-CNT1

EXAMINER SIGNATURE			
Examiner Signature	/PAUL J HOLLAND/	Date Considered	01/02/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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Art Unit	1656
Examiner Name	Paul J. Holland
Attorney Docket Number	50091-CNT1

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Crissa A. Cook/	Date (YYYY-MM-DD)	2019-12-19
Name/Print	Crissa A. Cook	Registration Number	64054

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Bibliographic Data

Application No: 15/188,478

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☑ No ☐ Met After Allowance

Verified and Acknowledged: /PAUL J HOLLAND/ (Examiner's Signature) ____ (Initials)

Title: CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
06/21/2016	530	1656	50091-CNT1
RULE			

APPLICANTS

Ventria BioScience Inc., Fort Collins, CO,

INVENTORS

Steven Clyde Pettit Fort Collins, CO, UNITED STATES

Mary Ann Michelle Fernandez Santos Elk Grove, CA, UNITED STATES

Ning Huang Davis, CA, UNITED STATES

CONTINUING DATA

This application is a CON of 12708462 02/18/2010ABN

12708462 has PRO of 61154204 02/20/2009

FOREIGN APPLICATIONS

IF REQUIRED, FOREIGN LICENSE GRANTED**

06/30/2016

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(Not for submission under 37 CFR 1.99)

Application Number	15188478
Filing Date	2016-06-21
First Named Inventor	Steven Clyde Pettit
Art Unit	1656
Examiner Name	Paul J. Holland
Attorney Docket Number	50091-CNT1

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6733746		2004-05-11	Daley	
	2	4075197		1978-02-21	Schuck	
	3	4086222		1978-04-25	Lindquist	
	4	4093612		1978-02-27	Travis	
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	31	7585955		2009-09-08	SANG	
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	1	20110189751		2011-08-04	Michael E. Barnett	
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	14	20090181426		2009-07-16	DILEO	
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2]i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
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	Examiner Name	Paul J. Holland
	Attorney Docket Number	50091-CNT1

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EXAMINER SIGNATURE

Examiner Signature	/PAUL J HOLLAND/	Date Considered	01/02/2020

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Crissa A. Cook/	Date (YYYY-MM-DD)	2019-12-19
Name/Print	Crissa A. Cook	Registration Number	64054

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Application Number	Filing Date	First Named Inventor	Atty. Docket No.	Confirmation No.
15188478	21-Jun-2016	Steven Pettit	50091-CNT1	8304

TITLE OF INVENTION :

CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS

Entity Status	Application Type	Art Unit	Class - Subclass	EXAMINER
Small	Utility under 35 USC 111(a)	1656	404000	PAUL HOLLAND

Issue Fee Due	Publication Due	Total Fee(s) Due	Date Due	Prev. Paid Fee
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○ Applicant changing to regular undiscounted fee status.
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6.Signature

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

Signature	/Crissa A. Cook/	**Date**	02-26-2020
Name	Crissa A. Cook	**Registration Number**	64054

Electronic Patent Application Fee Transmittal

Application Number:	15188478
Filing Date:	21-Jun-2016
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Perrit
Filer:	Crissa A. Cook/Brandy Goodman
Attorney Docket Number:	50091-CNT1

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPL ISSUE FEE	2501	1	500	500
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	500

Electronic Acknowledgement Receipt	
EFS ID:	38698514
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	26-FEB-2020
Filing Date:	21-JUN-2016
Time Stamp:	14:58:28
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Deposit Account	190522
Authorized User	Brandy Goodman
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	Web85b.pdf	46064	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	31748	no	2
Warnings:					
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New Applications Under 35 U.S.C. 111
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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Petta	50091-CNT1	8304

23589 7590 02/28/2020

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER
HOLLAND, PAUL J

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
02/28/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

uspatents@hoveywilliams.com

PTOL-90A (Rev. 04/07)

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Application No. : 15188478
Applicant : Pettit
Filing Date : 06/21/2016
Date Mailed : 02/28/2020

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

*A copy of this notice **MUST** be returned with the reply. Please address response to*
"Mail Stop Issue Fee, Commissioner for Patents,
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/Shirley Winslow/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 15188478

IDENTIFICATION OF APPLICATION DEFICIENCIES IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012

[X] Applicant must provide legible text for the following item(s).

- [X] Specification filed 07/09/2018, page(s) 101-103 (illegible tables).
- [ ] Claims filed , claim(s) .
- [ ] Other: .

[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(c) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Docket No.: 50091-CNT
Steven Clyde PETTIT	Customer No.: 23589
Serial No.: 15/188,478	Group Art Unit No.: 1656
Filed: June 21, 2016	Confirmation No.: 8304
CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS	Examiner: Paul J. HOLLAND

Mail Stop ISSUE FEE
Attn: Shirley Winslow
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDMENT

In response to the Notice to File Corrected Application Papers dated February 28, 2020, amendment and reconsideration of the above-referenced application is requested.

Amendments to the Specification begin on page 2.

Remarks begin on page 3.

Amendments to the Specification:

Please replace the pending specification with the Substitute Specification, submitted herewith.

Remarks:

In response to the Notice, the Specification is amended herein via a Substitute Specification. The issue fee has already been paid.

Applicant notes that there are no markings in the submitted copy of the Substitute Specification, because **the only changes made in the application were to adjust the font** of the text in the Tables on pages 101-103. No other changes have been made to the Specification.

No new matter is added in this Amendment.

Should the Examiner have any questions, please contact the undersigned. The Commissioner is hereby authorized to charge any additional fees associated with this communication or credit any overpayment to Deposit Account No. 19-0522.

Respectfully submitted,

/Crissa A. Cook/

By _____

Crissa A. Cook, Reg. No. 64,054
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ATTORNEYS FOR APPLICANT(S)

Cell Culture Media Containing Combinations of Proteins

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Serial No. 12/798,462, filed February 18, 2010, which claims the benefit of US provisional patent application No. 61,154,204, filed February 20, 2009, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

BACKGROUND OF THE INVENTION

Cell culture techniques allow animal or plant cells that are removed from tissues to grow when supplied with the appropriate nutrients and conditions. The cells are capable of dividing and can continue to grow until limited by culture variables such as nutrient depletion or toxic buildup (Butler, M. & Jenkins, H., "Nutritional aspects of growth of animal cells in culture," J of Biotechnol. (1989) 12: 97-110). Cell culture techniques have a number of applications including investigation of the normal physiology or biochemistry of cells (Balaban, B. & Urman, B., "Embryo culture as a diagnostic tool," Reprod. Biomed. Online (2003) 7(6): 671-82), testing the effect of various chemical compounds or drugs on specific cell types (Farkas, D. & Tannenbaum, S.R., "In vitro methods to study chemically-induced hepatotoxicity: a literature review," Curr. Drug Metab. (2005) 6(2): 111-25), studying the sequential or parallel combination of various cell types to generate artificial tissues (Wang et al., "Cartilage tissue engineering with silk scaffolds and human articular chondrocytes," Biomaterials (2006)), and synthesizing valuable biologics from large scale cell cultures (Zeilinger et al., "Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions," Altern. Lab Anim. (2002) 30(5): 525-38). Cell culture techniques have also been used for in vitro fertilization (Blake et al., "Protein supplementation of human IVF culture media," J Assist Reprod. Genet. (2002) 19(3): 137-43; Bungum et al., "Recombinant human albumin as protein source in culture media used for IVF: a prospective randomized study," Reprod. Biomed. Online (2002) 4(3): 233-6), stem cell research (Conley et al., "Derivation, propagation and differentiation of human embryonic stem cells," Int. J Biochem Cell Biol. (2004) 36(4): 555-67), vaccine production (Chuang et al., "Pharmaceutical strategies utilizing recombinant human albumin," Pharm. Res. (2002) 19(5): 569-77; GlaxoSmithKline, HAVRIX® (Hepatitis A Vaccine, Inactivated) - Prescribing Information

(2005), available at us.gsk.com/products/assets/us_havrix.pdf; Innis et al., "Protection against hepatitis A by an inactivated vaccine," JAMA (1994) 271(17): 1328-34; Merck, PROQUAD® - Measles, Mumps, Rubella, and Varicella (Oka/Merck) Virus Vaccine Live - Prescribing Information (2005), available at www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf; Litwin, J., "The growth of Vero cells in suspension as cell-aggregates in serum-free media," Cytotechnology (1992) 10(2): 169-74), tissue engineering including artificial skin (Atala, A., "Future perspectives in reconstructive surgery using tissue engineering," Urol. Clin. North Am. (1999) 26(1): 157-65, ix-x; Sher, et al., "Targeting perlecan in human keratinocytes reveals novel roles for perlecan in epidermal formation," J. Biol. Chem. (2006) 281(8): 5178-87) and organs (Neronov et al., "Integrity of endothelium in cryopreserved human cornea," Cryo Letters (2005) 26(2): 131-6; Han, et al., "Interleukin-1 alpha-induced proteolytic activation of metalloproteinase-9 by human skin," Surgery (2005) 138(5): 932-9) and gene and cell therapy (Chadd, H.E. & Chamow, S.M., "Therapeutic antibody expression technology," Curr. Opin. Biotechnol. (2001) 12(2): 188-94).

Biologics encompass a broad range of cell products, and include specific proteins or viruses that require animal cells for propagation. For example, therapeutic proteins such as monoclonal antibodies can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures (Dewar et al., "Industrial implementation of in vitro production of monoclonal antibodies, Ilar J. (2005) 46(3): 307-13). The number of such commercially valuable biologics has increased rapidly over the last decade and has led to the present widespread interest in mammalian cell culture technology (Mizrahi, A., "Biologicals produced from animal cells in culture – an overview," Biotechnol. Adv. (1988) 6(2): 207-20).

The major advantage of using cell culture for any of the above applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells. The need for cell culture, especially at large scale, became apparent with the need for viral vaccines. Major epidemics of polio in the 1940s and 1950s promoted efforts to develop an effective vaccine. In 1949, it was shown that poliovirus could be grown in cultures of human cells, which led to considerable interest in the development of large quantities of the polio vaccine using cell culture (Ligon, B.L., "Thomas Huckle Weller MD: Nobel Laureate and research pioneer in poliomyelitis, Varicella-zoster virus, cytomegalovirus, rubella, and other infectious diseases," Semin. Pediatr. Infect. Dis. (2002) 13(1): 55-63). The polio vaccine, produced from de-activated virus, became one of the first commercial products of cultured animal cells (Furesz, J., "Developments in the production and quality control of poliovirus vaccines – Historical perspectives," Biologicals (2006))

Due to the safety and ethical considerations associated with the use of animal-derived cell culture media components, efforts have been made to provide alternative sources for cell culture media and media components

Published PCT Appl. No. WO 2007/002762, which was based on U.S. Provisional Appl. No. 60/694,236, filed June 28, 2006, relates to recombinant production of components of cell culture media using plant cells, and cell culture media containing such recombinant proteins. The entire contents of this application are incorporated herein by reference.

SUMMARY OF THE INVENTION

Accordingly, it is an objective of the invention to provide cell culture media or media supplements containing combinations of protein components, which when used in cell culture, result in improved growth and/or productivity of cultured cells.

One embodiment of the invention is a cell culture media including a cell culture media base, and two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

Yet another embodiment of the invention is a cell culture media including a cell culture media base, albumin, and at least one additional protein selected from the group consisting of growth factors, lactoferrin, transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the growth factors that may be provided in the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

Another embodiment of the invention is a cell culture media including a cell culture media base, lactoferrin, and at least one additional protein selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, fibroblast growth factors, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin.

According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media are plant-produced heterologous proteins.

According to further embodiments, a preferred combination of proteins that may be provided in the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides methods for producing supplemented cell culture media, including providing a cell culture media base; providing two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin; and combining the two or more proteins with the cell culture media base to form the supplemented cell culture media.

According to further embodiments, the proteins used in the methods for producing cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for producing cell culture media are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be provided in the methods of producing the cell culture media are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors,

transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of producing the cell culture media includes lactoferrin and albumin

Another embodiment of the invention provides a method for culturing cells, including providing a cell culture media base, supplementing the cell culture media base with two or more proteins selected from the group consisting of growth factors, lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, and alpha-lactalbumin to form a supplemented cell culture media, and introducing cells to be cultured into the supplemented cell culture media. The cells grown in the supplemented cell culture media exhibit improved growth characteristics as compared to cells grown in an unsupplemented cell culture media.

According to further embodiments, the proteins used in the methods for culturing cells are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, preferably two or more of the proteins used in the methods for culturing cells are plant-produced heterologous proteins, and more preferably all of the proteins used in the methods for culturing cells are plant-produced heterologous proteins.

According to further embodiments, the growth factors that may be used in the methods for culturing cells are selected from the group consisting of epidermal growth factors, keratinocyte growth factors, insulin-like growth factors, intestinal trefoil factors, transforming growth factors, granulocyte colony-stimulating factors, nerve growth factors, and fibroblast growth factors.

According to further embodiments, a preferred combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, by culturing the cultured cell in the cell culture media of the present invention

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a supplement comprising; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100,000, to about 1 to 1,000.

In one aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01 % wt / wt of a heat shock protein. In aspect of this embodiment, the heat shock protein is a rice heat shock protein. In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein. In another aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / OsI_37938, and AAB63469

In another aspect of any of the claimed supplements, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt/wt HSP70.

In another aspect of any of the claimed supplements, the transferrin related protein is lactoferrin. In another aspect of any of the claimed supplements, the transferrin related protein is transferrin. In one aspect, of any of the claimed supplements the lactoferrin is human. In one aspect, of any of the claimed supplements the transferrin is human. In one aspect, of any of the claimed supplements the lactoferrin is recombinant. In one aspect, of any of the claimed supplements the lactoferrin is derived from plasma. In one aspect, of any of the claimed supplements the transferrin is recombinant. In one aspect, of any of the claimed supplements the transferrin is derived from plasma. In one aspect, of any of the claimed supplements the IGF-1 is human. In one aspect, of any of the claimed supplements the IGF-1 is recombinant.

In another aspect of any of the claimed supplements, the ratio of transferrin to albumin about 1 to 50, to about 1 to 200. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 75, to about 1 to 180 In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 80, to about 1 to 120. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 1000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 2000. In another aspect of any of the claimed supplements. the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 3000. In another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50. to about 1 to 4000. in another aspect of any of the claimed supplements, the ratio of the transferrin to the albumin about 1 to 50, to about 1 to 5000.

In another aspect of any of the claimed supplements. the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0 5, to about 1 to 2 In another aspect of any of the claimed supplements, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and a

transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media, wherein the supplement comprises. a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5.

In another embodiment, the invention includes a method for reducing the accumulation of Lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein. wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method of reducing the time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of

albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein, wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free media, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin. and iii) less than about 2 % aggregated albumin. and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin, and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5.

In another embodiment, the invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the cell culture medium wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a

plant. ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5

In another embodiment, the invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection, wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0 5

In another embodiment, the invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing; wherein the supplement comprises; a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin, and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 1000.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises a mixture of recombinant albumin and a transferrin related protein; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin, / mg of albumin. and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of transferrin related protein to albumin of about 1 to 5000, to about 1 to 0.5. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated

bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In another embodiment, the invention includes a method for improving the yield of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture wherein the supplement comprises, a mixture of recombinant albumin and an insulin related growth factor; wherein the recombinant albumin is i) produced in a plant, ii) has less than about 1 EU of endotoxin / mg of albumin; and iii) less than about 2 % aggregated albumin; and wherein the supplement comprises a ratio (wt/wt) of insulin related growth factor to albumin of about 1 to 100000, to about 1 to 5000. In one aspect of this method, the viability of the cell culture is increased. In one aspect of this method the product is a protein or cell associated bacteria. In another aspect, the protein is an antibody. In one aspect, the protein is a recombinant protein, in another aspect, the protein is a monomer, in another aspect, the protein is a multimeric protein, in another aspect, and the antibody is full length. In another aspect, the antibody is a single chain antibody.

In one aspect of any of these methods, the supplement further comprises insulin. In one aspect of any of these methods, the supplement further comprises IGF-1. In another aspect, the supplement further comprises selenium and ethanolamine.

In another aspect of any of the claimed methods, the cells are tissue culture cells. In another aspect, the cells are CHO cells. In another aspect the cells are hybridoma cells. In another aspect the cells are vero cells. In another aspect the cells are sorted by flow cytometry. In another aspect the cells are primary cells. In one aspect, the primary cells are embryonic stem cells. In another aspect the primary cells are B-cell derived. In another aspect the cells the primary cells are T-cell derived. In another aspect the cells are B-cells or B-cell derived. In another aspect the cells are T-cells or T-cell derived. In another aspect the cells. In another aspect the cells are isolated by a micro fluidic device. In another aspect the cells are isolated by single-cell subcloning

In one aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt / wt of a heat shock protein In aspect of this embodiment, the heat shock protein is a rice heat shock protein In another aspect, the heat shock protein is selected from the group consisting of Rice HSP70 genes, and rice endosperm lumenal binding protein In another

aspect, heat shock protein is selected from the group consisting of Rice (gb|ACJ54890.1|), EEC69073 / Osl_37938, and AAB63469.

In another aspect of any of the claimed methods, the recombinant albumin comprises at least about 0.01% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.04% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.06% wt/wt HSP70. In another aspect, the recombinant albumin at least about 0.08% wt/wt HSP70. In another aspect, the recombinant albumin comprises at least about 0.1% wt /wt HSP70

In another aspect of any of the claimed methods, the transferrin related protein is lactoferrin. In another aspect of any of the claimed methods the transferrin related protein is transferrin. In one aspect, of any of the claimed methods the lactoferrin is human. In one aspect, of any of the claimed methods the transferrin is human. In one aspect, of any of the claimed methods the lactoferrin is recombinant. In one aspect, of any of the claimed methods the transferrin is recombinant.

In another aspect of any of the claimed methods, the ratio of transferrin to albumin is about 1 to 50, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 75, to about 1 to 180. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 80, to about 1 to 120. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 120, to about 1 to 200. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 200, to about 1 to 1000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 1000, to about 1 to 2000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 2000, to about 1 to 3000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 3000, to about 1 to 4000. In another aspect of any of the claimed methods, the ratio of the transferrin to the albumin is about 1 to 4000, to about 1 to 5000.

In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 3, to about 1 to 0.33. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.5, to about 1 to 2. In another aspect of any of the claimed methods, the ratio of lactoferrin to albumin is about 1 to 0.8, to about 1 to 1.2.

In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 100 mg /L and 400 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 400 mg /L and 800 mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 800 mg /L and 1000mg/ L. In another aspect of any of the claimed methods comprising lactoferrin, the lactoferrin is added to a final concentration of between about 1000 mg /L and 2000 mg/ L.

In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 1ug /L and 50 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 2 ug /L and 10 ug/ L In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 3 ug /L and 8 ug/ L. In another aspect of any of the claimed methods comprising transferrin, the transferrin is added to a final concentration of between about 4 ug /L and 6 ug/ L.

In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 100 mg /L and 2000 mg/ L. In another aspect of any of the claimed methods the recombinant albumin is added to a final concentration of between about 200 mg /L and 1000 mg/ L.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 30 % compared to the summed effect of recombinant albumin and transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 40 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 50 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 60 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 70 % compared to the summed effect of recombinant albumin or transferrin related protein alone when measured under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 80 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 90 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 100 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

In another aspect of any of the claimed methods the improvement in cell viability is greater than 150 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions

In another aspect of any of the claimed methods the improvement in cell viability is greater than 200 % compared to the summed effect of recombinant albumin or transferrin related protein when added alone under the same culture conditions.

Other novel features and advantages of the present invention will become apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

BRIEF DESCRIPTION OF THE FIGURES

For a more complete understanding of the present invention, the needs satisfied thereby, and the objects, features, and advantages thereof, reference now is made to the following description taken in connection with the accompanying drawings.

FIG. 1A is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1B is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22 5%) nucleotide changes

FIG. 1C is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 1D is a comparison of the codon-optimized human lactoferrin sequence ("cod opt lac") with a native human lactoferrin sequence ("native lac"), aligned to show 693 codons, with 413 (59.6%) codon changes and 467 (22.5%) nucleotide changes.

FIG. 2 is a plasmid map of the 5,817 bp plasmid, API164 (GT1-Lac), showing an expression cassette for human lactoferrin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human lactoferrin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 3 shows the results of a SDS-PAGE analysis for the expression of recombinant human lactoferrin. Total proteins from rice seed extracts were suspended in Laemli sample buffer, run on a gradient gel and stained with Coomassie blue. Lane 1 is the molecular weight marker. Lanes 3 to 6 are purified human derived lactoferrin (Sigma Chemical, USA). Lanes 8 to 13 are single seed extracts from homozygous independent transgenic rice lines and lane 14 is a seed extract from non-transformed rice variety Taipei 309.

FIG. 4 is a stable expression of recombinant human lactoferrin in transgenic rice grains from R_2 through R_{10} generations. Total soluble proteins from 1 g of brown rice flour was extracted with 40 ml of extraction buffer and clarified by centrifugation. The extract was analyzed via ELISA. Extraction was repeated three times and standard deviation is shown as an error bar.

FIG. 5A shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5B shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 5C shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot)

FIG. 5D shows DNA and protein sequence of fusion between Gt1 signal peptide (Gt1 SP) and codon-optimized human albumin (OPTIMIZED HSA) sequence based on native protein sequence derived from P02768 (Swiss-Prot).

FIG. 6 is a plasmid map of the 5,496 bp plasmid, API504 (GT1-HSA), showing an expression cassette for human albumin and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 7 is a plasmid map of the 12,388 bp plasmid, API508 (JH/GT1-HSA), showing an expression cassette for human serum albumin (HSA) and containing a Gt1 promoter, a Gt1 signal peptide, codon optimized human albumin, a Nos terminator and a kanamycin resistance selectable marker.

FIG. 8A-FIG. 8E Shows a comparison by HPLC size exclusion chromatography of recombinant HSA produced from rice compared to other sources of albumin and methods of purification. **FIG. 8A** shows the chromatogram for a serum derived (non-recombinant HSA). **FIG. 8B** shows the chromatogram for a rice recombinant HSA (Cellastim P0107) made using the "old process" B000 for purification. **FIG. 8C** shows the chromatogram for a rice recombinant HSA (Cellastim P0171) made using the "new process" B0000C for purification. **FIG. 8D** shows an overlay of the chromatograms for the serum derived albumin (1A; dotted line) and Cellastim HSA prepared using the new process ((1C; solid line). **FIG. 8E** shows an overlay of the chromatograms for HSA prepared using the old process B000 (Cellastim P0107) (1B, dotted line) and HSA prepared using the new process B0000C (Cellastim P0171) (1C; solid line).

FIG. 9A-FIG. 9B Shows a comparison by SDS PAGE analysis of recombinant albumin produced from rice compared to other sources of HSA and methods of purification **FIG. 9A** shows a comparison of Cellastim P0171albumin and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **FIG. 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

FIG. 10A: Shows a comparison of the effects of yeast recombinant HSA (Cellprime), human plasma derived HSA, (Seracare) and plant recombinant HSA (Cellastim P0171) with respect to their ability to promote cell growth and viability. **FIG. 10B** shows a comparison of the endotoxin levels in batches of albumin produced using the old (B000) and new processes (B0000C) for recombinant albumin production. **FIG. 10C** shows a comparison of cell growth and viability of cells grown in the presence of the Cellastim HSA produced using the old (B000) and new processes (B0000C) for recombinant albumin production.

FIG. 11: Shows a western blot of two independent experiments showing that plant derived recombinant HSA comprises proteins that can be selectively concentrated by an ATP affinity column (Lanes 4 & 9) and react against an anti-HSP antibody. Lanes 3 and 7 show 10 ug of starting material; Lanes 4 and 9 show the fractions after elution and concentration from the ATP affinity column; Lanes 5 and 8 show the wash through after elution with ATP, and lane 10 is a hsp positive control. (See Example 9 for details).

FIG. 12: Shows a comparison of the cell growth and viability effect of Cellastim recombinant albumin after passing the albumin produced using the new process over an ATP affinity column to remove heat shock proteins. (See text for details)

FIG. 13: Shows a comparison of the effect of 1 g/L recombinant plant derived human serum albumin (rHSA) in combination with transferrin (pTF) compared to the same concentration of plasma derived human serum albumin (pHSA) in combination with transferrin (pTF) on the production of antibody by hybridoma cells in culture.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

In order that the present disclosure may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description. Unless otherwise indicated, all terms used herein have the meanings given below, or are generally consistent with same meaning that the terms have to those skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds. (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands, for definitions and terms of the art.

The term "**about**" or "**approximately**" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviations, per practice in the art. Alternatively, "about" with respect to the compositions can mean plus or minus a range of up to 20%, preferably up to 10%, more preferably up to 5%.

The term "**antigen-binding fragment**" refers to a polypeptide portion of an immunoglobulin or antibody that binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). Binding fragments can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', $F(ab')_2$, Fabc, Fv, single chains, and single-chain antibodies.

The term "**apoptosis**" ("normal" or "programmed" cell death) refers to the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Apoptosis is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide"). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine dependent tissue atrophy. Apoptosis may also be triggered in cells grown under tissue culture conditions in response to stress. Cells undergoing apoptosis show characteristic morphological and biochemical features, which can be readily measured and quantified. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed "secondary necrosis".

As used herein, the terms "**cell**," "**cells**," "**cell line**," "**host cell**," and "**host cells**," are used interchangeably and, encompass plant, and animal cells and include invertebrate, non-mammalian vertebrate and mammalian cells. All such designations include cell populations and progeny. Thus, the terms "transformants" and "transfectants" include the primary subject cell and cell lines derived therefrom without regard for the number of transfers. Exemplary non-mammalian vertebrate cells include, for example, avian cells, reptilian cells and amphibian cells.

Exemplary invertebrate cells include, but are not limited to, insect cells such as, for example, caterpillar (Spodoptera frugiperda) cells, mosquito (Aedes aegypti) cells, fruitfly (Drosophila melanogaster) cells, Schneider cells, and Bombyx mori cells. See, e.g., Luckow et al., Bio/Technology 6:47-55 (1988) The cells may be differentiated, partially differentiated or undifferentiated, e.g. stem cells, including embryonic stem cells and pluripotent stem cells. Additionally tissue samples derived from organs or organ systems may be used according to the invention. Exemplary mammalian cells include, for example, cells derived from human, non-human primate, cat, dog, sheep, goat, cow, horse, pig, rabbit, rodents including mouse, hamster, rat and guinea pig and any derivatives and progenies thereof

The terms "**cell culture**," or "**tissue culture**" refer to cells grown in suspension or grown adhered to a variety of surfaces or substrates in vessels such as roller bottles, tissue culture flasks, dishes, multi-well plates and the like. Large scale approaches, such as bioreactors, including adherent cells growing attached to microcarriers in stirred fermentors, are also encompassed by the term "cell culture." Moreover, it is possible not only to culture contact-dependent cells, but also to use suspension culture techniques in the methods of the claimed invention. Exemplary microcarriers include, for example, dextran, collagen, plastic, gelatin and cellulose and others as described in Butler, Spier & Griffiths, Animal cell Biotechnology 3:283-303 (1988). Porous carriers, such as, for example, Cytoline™ or Cytopore™, as well as dextran-based carriers, such as DEAE-dextran (Cytodex 1™ quaternary amine-coated dextran (Cytodex™) or gelatin-based carriers, such as gelatin-coated dextran (Cytodex 3™) may also be used. Cell culture procedures for both large and small-scale production of proteins are encompassed by the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, with or without microcarriers, and operated alternatively in a batch, fed-batch, or perfusion mode.

The terms "**cell culture medium**," "**cell culture media**," and "**culture medium**" refer to the solutions used for growing, storing, handling and maintaining cells and cell lines. Such solutions generally include various factors necessary for cell attachment, growth, and maintenance of the cellular environment. For example, a typical solution may include a basal media formulation, various supplements depending on the cell type and, occasionally, antibiotics. In some embodiments, a solution may include at least one component from one or more of the following categories. 1) an energy source, usually in the form of a carbohydrate such as glucose; 2) all essential amino acids, and usually the basic set of twenty amino acids plus cystine; 3) vitamins and/or other organic compounds required at low concentrations; 4) free fatty acids; and 5) trace

elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range. The solution may optionally be supplemented with one or more components from any of the following categories: 1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor; 2) salts and buffers as, for example, calcium, magnesium, phosphate, Tris, HEPES, and sodium bicarbonate; 3) nucleosides and bases such as, for example, adenosine and thymidine, hypoxanthine; and 4) protein and tissue hydrolysates. In general, any suitable cell culture medium may be used. The medium may be comprised of serum, e.g. fetal bovine serum, calf serum or the like. Alternatively, the medium may be serum free, animal free, or protein free.

The term "**cell culture media component**" or "cell culture component" refers to any heterologous proteins used as a supplement for cell culture media.

The term "**cell culture media ingredient**" includes Cell culture media components, proteins, peptides, hormones, carbohydrates, amino acids, lipids, vitamins, antibiotics, organic and inorganic salts.

The term "**cell culture media supplement**" refers to a combination of one or multiple cell culture media components, with or without other ingredients, for addition to cell culture media.

The term "**cell culture media base**" or "**basal media**" refers to a cell culture media that may contain, for example, any or all of the following components: proteins, peptides, lipids, carbohydrates, amino acids, organic and/or inorganic salts, buffers (e.g., bicarbonate), vitamins, hormones, antibiotics, and pH indicators (e.g., phenol red). Examples of cell culture media bases that can be used in accordance with the present invention include: Dulbecco's Modified Eagle's Medium (DME), Ham's Nutrient Mixtures, MCDB Media, Minimum Essential Medium Eagle, RPMI Media, Ames' Media, BGJb Medium (Fitton-Jackson Modification), Click's Medium, CMRL-1066 Medium, Fischer's Medium, Glascow Minimum Essential Medium (GMEM), Iscove's Modified Dulbecco's Medium (IMDM), L-15 Medium (Leibovitz), McCoy's 5A Modified Medium, NCTC Medium, Swim's S-77 Medium, Waymouth Medium, and William's Medium E. The cell culture media base may be supplemented using a cell culture media component or a cell culture media supplement.

The term "**cell lineage**" when referring to a stem cell culture refers to all of the stages of the development of a cell type, from the earliest precursor cell to a completely mature cell (i.e. a

specialized cell).

The terms "**cell viability**" or "**viability**" refers to relative amounts of living and dead cells, present with a population of cells at any given time Cell viability may be determined by measuring the relative numbers of living and dead cells in any given sample of the population. Cell viability may also be estimated by measuring the rate of cell proliferation of the entire population which represents the overall balance of the rates of cell growth and cell death. Rates of cell growth may also be directly measured, by counting the number of cells, and by using any number of commercially available cell proliferation assays which directly scores the rate of cell growth.

"**Conditioned medium**" refers to a cell culture medium that is obtained from a culture of a feeder cell on which embryonic stem cells can be cultured and maintained in a pluripotent state. The feeder cell depletes the conditioned medium of some components, but also enriches the medium with cell-derived material, probably including small amounts of growth factors. The term "feeder cell factor" as used herein means the cell-derived material that is released into the conditioned medium by the feeder cell. The cell factor that is released into the cell culture medium is useful in enhancing the growth of embryonic stem cells, or in the maintenance of the embryonic stem cell in a pluripotent state. The feeder cell factor can be identified and purified using techniques that are known to one skilled in the art, and are described herein

The phrase "**conservative amino acid substitution**" or "**conservative mutation**" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. and R. H. Schirmer, Principles of Protein Structure, Springer-Verlag)

Examples of amino acid groups defined in this manner include: a "charged / polar group," consisting of Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic, or cyclic group," consisting of Pro, Phe, Tyr and Trp; and an "aliphatic group" consisting of Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys

Within each group, subgroups can also be identified, for example, the group of charged / polar

amino acids can be sub-divided into the sub-groups consisting of the "positively-charged sub-group," consisting of Lys, Arg and His; the negatively-charged sub-group," consisting of Glu and Asp, and the "polar sub-group" consisting of Asn and Gln. The aromatic or cyclic group can be sub-divided into the sub-groups consisting of the "nitrogen ring sub-group," consisting of Pro, His and Trp; and the "phenyl sub-group" consisting of Phe and Tyr. The aliphatic group can be sub-divided into the sub-groups consisting of the "large aliphatic non-polar sub-group," consisting of Val, Leu and Ile; the "aliphatic slightly-polar sub-group," consisting of Met, Ser, Thr and Cys; and the "small-residue sub-group," consisting of Gly and Ala.

Examples of conservative mutations include substitutions of amino acids within the sub-groups above, for example, Lys for Arg and vice versa such that a positive charge can be maintained; Glu for Asp and vice versa such that a negative charge can be maintained; Ser for Thr such that a free -OH can be maintained, and Gln for Asn such that a free -NH_2 can be maintained.

The term "**cytotoxicity**" refers to the cell killing property of a chemical compound (such as a chemical or protein contaminant, detergent, or toxin). In contrast to necrosis and apoptosis, the term cytotoxicity need not necessarily indicate a specific cellular death mechanism.

As used herein, the term "**decrease**" or the related terms "**decreased**," "**reduce**" or "**reduced**" refers to a statistically significant decrease. For the avoidance of doubt, the terms generally refer to at least a 10% decrease in a given parameter, and can encompass at least a 20% decrease, 30% decrease, 40% decrease, 50% decrease, 60% decrease, 70% decrease, 80% decrease, 90% decrease, 95% decrease, 97% decrease, 99% or even a 100% decrease (i.e., the measured parameter is at zero).

As used herein, the terms "**develop**", "**differentiate**" and "**mature**", as used to describe a stem cell, refer to the progression of a cell from the stage of having the potential to differentiate into at least two different cellular lineages to becoming a specialized and terminally differentiated cell. Such terms can be used interchangeably for the purposes of the present application.

The term "**expression**" as used herein refers to transcription and/or translation of a nucleotide sequence within a host cell. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired polypeptide encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, in situ hybridization to cellular RNA or by PCR. Proteins encoded

by a selected sequence can be quantified by various methods including, but not limited to, e.g., ELISA, Western blotting, radioimmunoassays, immunoprecipitation, assaying for the biological activity of the protein, or by immunostaining of the protein followed by FACS analysis.

"Expression control sequences" are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, internal ribosome entry sites (IRES) and the like, that provide for the expression of a coding sequence in a host cell. Exemplary expression control sequences are described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego Calif. (1990).

The term "**feeder cell**" refers to a culture of cells that grows in vitro and secretes at least one factor into the culture medium, and that can be used to support the growth of another cell of interest in culture. As used herein, a "**feeder cell layer**" can be used interchangeably with the term "feeder cell." A feeder cell can comprise a monolayer, where the feeder cells cover the surface of the culture dish with a complete layer before growing on top of each other, or can comprise clusters of cells

The term "**growth phase**" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are dividing at a constant rate. During this phase, cells are cultured for a period of time, and under such conditions that cell growth is maximized. The determination of the growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. "Period of time and under such conditions that cell growth is maximized" and the like, refer to those culture conditions that, for a particular cell line, are determined to be optimal for cell growth and division. During the growth phase, cells are cultured in nutrient medium containing the necessary additives usually at about 30-40 ° C., generally about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line, for instance a mammalian cell.

The term "**heterologous DNA**" refers to DNA which has been introduced into a cell that is derived from another source, or which is from the same source but is located in a different (i.e. non native) context. The term "**heterologous protein**" or "**recombinant protein**" refers to a protein encoded in all or part by heterologous DNA, or a protein that is expressed from expression control sequences (such as a promoter, or enhancer) created in whole or part by the heterologous DNA which activates the expression of an endogenous gene.

The term "**homology**" describes a mathematically based comparison of sequence similarities

which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes. Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used.

The term "**homologous**" refers to the relationship between two proteins that possess a "common evolutionary origin", including proteins from superfamilies (e.g., the immunoglobulin superfamily) in the same species of animal, as well as homologous proteins from different species of animal (for example, myosin light chain polypeptide, etc., see Reeck et al., *Cell*, 50:667, 1987). Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term "**growth factor**" refers to Amphiregulin, Angiopoietin, Betacellulin, (Bone Morphogenic protein-13, Bone Morphogenic protein-14, Bone Morphogenic protein-2, Human BMP-3, Bone Morphogenic protein-4, Human BMP-5, Bone Morphogenic protein-6, Bone Morphogenic protein-7, Human CD135 Ligand / Flt-3 Ligand, Human Granulocyte Colony Stimulating Factor (G-CSF), Human Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Human Macrophage Colony Stimulating Factor (M-CSF), Human Cripto-1, Human CTGF (Connective tissue growth factor), Human EGF (Epidermal Growth Factor), Human EG-VEGF (Endocrine-Gland-Derived Vascular Endothelial Growth Factor), Human Erythropoietin (EPO), Human FGF (Fibroblast Growth Factors 1-23), Human GDF-11, Human GDF-15, Human GDF-8, Human Growth Hormone Releasing Factor (GHRF, GRF, GHRH, Growth Hormone Releasing Hormone), Human Heparin Binding Epidermal Growth Factor (HB-EGF), Human Hepatocyte Growth Factor (HGF), Human Heregulin beta 1, Human insulin, Human IGF-1 (Insulin-like Growth Factor-1), Human IGF-2 (Insulin-like Growth Factor-2), Human IGFBP-1 (Insulin-like Growth Factor Binding Protein 1), Human IGFBP-3 (Insulin-like Growth Factor Binding Protein 3), intestinal trefoil factor (ITF), Human keratinocyte growth factors 1 & 2, Human Leukemia

Inhibitory Factor (LIF), Human MSP, Human Myostatin, Human Myostatin, pro (propeptide), Human NRG1, Human NGF, Human Oncostatin M, Human Osteoblast Specific Factor 1 (OSF-1, Pleiotrophin), Human PD-ECGF (Platelet-derived endothelial cell growth factor), Human PDGF, Human PlGF, Human Placental Growth Factor 1 (PLGF1), Human Placental Growth Factor 2 (PLGF2), Human SCGF-a (Stem Cell Growth Factor-alpha), Human SCGF-b (Stem Cell Growth Factor-beta), Human Stem Cell Factor (SCF) / CD117 Ligand, Human Thrombopoietin (TPO, THPO), Human Transforming Growth Factor, Human TGF-alpha (Transforming Growth Factor-alpha, TGFa), Human TGF-beta 1 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 1.2 (Transforming Growth Factor-beta1, TGFb), Human TGF-beta 2 (Transforming Growth Factor-beta2, TGFb), Human TGF-beta 3 (Transforming Growth Factor-beta3, TGFb), Human VEGF (Vascular Endothelial Growth Factor), Human VEGF-121, Human VEGF-165, and Human VEGF-A

As used herein, "**identity**" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds , Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991, and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). The well known Smith Waterman algorithm can also be used to determine identity.

The terms "**immunoglobulin**" or "**antibody**" (used interchangeably herein) refers to a protein typically having a basic four-polypeptide chain structure consisting of two heavy and two light chains, the chains being stabilized, for example, by interchain disulfide bonds, which has the

ability to specifically bind antigen. The term "single-chain immunoglobulin" or "single-chain antibody" (used interchangeably herein) refers to a protein having a two-polypeptide chain structure consisting of a heavy and a light chain, the chains being stabilized, for example, by interchain peptide linkers, which has the ability to specifically bind antigen. The term "domain" refers to a globular region of a heavy or light chain polypeptide comprising peptide loops (e.g., comprising 3 to 4 peptide loops) stabilized, for example, by beta-pleated sheet and/or intrachain disulfide bond. Domains are further referred to herein as "constant" or "variable", based on the relative lack of sequence variation within the domains of various class members in the case of a "constant" domain, or the significant variation within the domains of various class members in the case of a "variable" domain. Antibody or polypeptide "domains" are often referred to interchangeably in the art as antibody or polypeptide "regions". The "constant" domains of an antibody light chain are referred to interchangeably as "light chain constant regions", "light chain constant domains", "CL" regions or "CL" domains. The "constant" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "CH" regions or "CH" domains). The "variable" domains of an antibody light chain are referred to interchangeably as "light chain variable regions", "light chain variable domains", "VL" regions or "VL" domains). The "variable" domains of an antibody heavy chain are referred to interchangeably as "heavy chain constant regions", "heavy chain constant domains", "VH" regions or "VH" domains). Immunoglobulins or antibodies may be monoclonal or polyclonal and may exist in monomeric or polymeric form, for example, IgM antibodies which exist in pentameric form and/or IgA antibodies which exist in monomeric, dimeric or multimeric form. The term "fragment" refers to a part or portion of an antibody or antibody chain comprising fewer amino acid residues than an intact or complete antibody or antibody chain. Fragments can be obtained via chemical or enzymatic treatment of an intact or complete antibody or antibody chain. Fragments can also be obtained by recombinant means. Exemplary fragments include Fab, Fab', F(ab')2, Fabc and/or Fv fragments.

As used herein, the term "**increase**" or the related terms "**increased**", "**enhance**" or "**enhanced**" refers to a statistically significant increase. For the avoidance of doubt, the terms generally refer to at least a 10% increase in a given parameter, and can encompass at least a 20% increase, 30% increase, 40% increase, 50% increase, 60% increase, 70% increase, 80% increase, 90% increase, 95% increase, 97% increase, 99% or even a 100% increase over the control value.

The term "**isolated**," when used to describe the cell culture components, i.e albumin, or transferrin related proteins disclosed herein, means protein that has been identified and

separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily however, isolated protein will be prepared by at least one purification step

The term "**maturation-specific protein promoter**" refers to a promoter exhibiting substantially up-regulated activity (greater than 25%) during seed maturation.

The term "**mature plant**" refers to a fully differentiated plant.

"**Markers**" as used herein, are nucleic acid or polypeptide molecules that are differentially expressed in a cell of interest. In this context, differential expression means an increased level for a positive marker and a decreased level for a negative marker. The detectable level of the marker nucleic acid or polypeptide is sufficiently higher or lower in the cells of interest compared to other cells, such that the cell of interest can be identified and distinguished from other cells using any of a variety of methods known in the art.

Cells expressing "**markers of pancreatic endocrine lineage**" refer to cells with positive gene expression for the transcription factor PDX-1 and at least one of the following transcription factors: NGN-3, NRx2.2, NRx6.1, NeuroD, Isl-1, HNF-3 beta, MAFA, Pax4, and Pax6. Cells expressing markers characteristic of the pancreatic cell lineage include pancreatic β cells.

Cells expressing "**markers characteristic of endoderm lineage**" as used herein refer to cells expressing at least one of the following markers: SOX-17, GATA-4, HNF-3 beta, GSC, Cer1, Nodal, FGF8, Brachyury, Mix-like homeobox protein, FGF4 CD48, eomesodermin (EOMES), DKK4, FGF17, GATA-6, CXCR4, C-Kit, CD99, or OTX2. Cells expressing markers characteristic of the definitive endoderm lineage include primitive streak precursor cells, primitive streak cells, mesendoderm cells and definitive endoderm cells.

Cells expressing pluripotency markers derived by the methods of the present invention express at least one of the following pluripotency markers selected from the group consisting of: ABCG2,

cripto, FoxD3, Connexin43, Connexin45, Oct4, SOX-2, Nanog, hTERT, UTF-1, ZFP42, SSEA-3, SSEA-4, Tral-60, and Tral-81.

Cells expressing "**markers characteristic of mesoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: CD48, eomesodermin (EOMES), SOX-17, DKK4, HNF-3 beta, GSC, FGF17, GATA-6.

Cells expressing "**markers characteristics of ectoderm lineage**" as used herein refers to a cell expressing at least one of the following markers: BMP-4, Noggin, Chordin, Otx2, Fox J3, Nestin, p63/TP73L, beta-III Tubulin.

"**Monocot seed components**" refers to carbohydrate, protein, and lipid components extractable from monocot seeds, typically mature monocot seeds

As used herein, the terms "**native**" or "**wild-type**" relative to a given cell, protein, polypeptide, nucleic acid, trait or phenotype, refers to the form in which that is typically found in nature.

The terms "**operably linked**" and "**operatively linked**," as used interchangeably herein, refer to the positioning of two or more nucleotide sequences or sequence elements in a manner which permits them to function in their intended manner. In some embodiments, a nucleic acid molecule according to the invention includes one or more DNA elements capable of opening chromatin and/or maintaining chromatin in an open state operably linked to a nucleotide sequence encoding a recombinant protein. In other embodiments, a nucleic acid molecule may additionally include one or more nucleotide sequences chosen from: (a) a nucleotide sequence capable of increasing translation; (b) a nucleotide sequence capable of increasing secretion of the recombinant protein outside a cell; and (c) a nucleotide sequence capable of increasing the mRNA stability, where such nucleotide sequences are operatively linked to a nucleotide sequence encoding a recombinant protein. Generally, but not necessarily, the nucleotide sequences that are operably linked are contiguous and, where necessary, in reading frame. However, although an operably linked DNA element capable of opening chromatin and/or maintaining chromatin in an open state is generally located upstream of a nucleotide sequence encoding a recombinant protein; it is not necessarily contiguous with it. Operable linking of various nucleotide sequences is accomplished by recombinant methods well known in the art, e.g using PCR methodology, by ligation at suitable restrictions sites or by annealing. Synthetic oligonucleotide linkers or adaptors can be used in accord with conventional practice if suitable restriction sites are not present.

A "**plant cell**" refers to any cell derived from a plant, including undifferentiated tissue (e.g., callus) as well as plant seeds, pollen, propagules, embryos, suspension cultures, meristematic regions, leaves, roots, shoots, gametophytes, sporophytes and microspores.

The terms "**polynucleotide**" and "**nucleic acid molecule**," used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. A nucleic acid molecule can take many different forms, e.g., a gene or gene fragment, one or more exons, one or more introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. As used herein, "DNA" or "nucleotide sequence" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides

The term "**pluripotent stem cell**" encompasses stem cells obtained from embryos, fetuses or adult tissues. In one preferred embodiment, the pluripotent stem cell is an embryonic stem cell. In another embodiment the pluripotent stem cell is a fetal stem cell, such as a primordial germ cell. In another embodiment the pluripotent stem cell is an adult stem cell.

As used herein, the term "**pluripotent**" refers to a cell capable of at least developing into one of ectodermal, endodermal and mesodermal cells. As used herein the term "pluripotent" includes cells that are totipotent and multipotent. As used herein, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. The term "multipotent" refers to a cell that is not terminally differentiated.

A "**promoter**" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. As used herein, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. A transcription initiation site (conveniently defined by mapping with nuclease S1) can be found within a promoter sequence, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters can often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine- Dalgarno sequences in addition to the -10 and -35 consensus sequences.

A large number of promoters, including constitutive, inducible and repressible promoters, from a variety of different sources are well known in the art. Representative sources include for example, viral, mammalian, insect, plant, yeast, and bacterial cell types, and suitable promoters from these sources are readily available, or can be made synthetically, based on sequences publicly available on line or, for example, from depositories such as the ATCC as well as other commercial or individual sources. Promoters can be unidirectional (i.e., initiate transcription in one direction) or bi-directional (i.e., initiate transcription in either a 3' or 5' direction). Non-limiting examples of promoters include, for example, the T7 bacterial expression system, pBAD (araA) bacterial expression system, the cytomegalovirus (CMV) promoter, the SV40 promoter, the RSV promoter, the rice endosperm specific glutelin (Gt1) promoter, CaMV35S viral promoter. Inducible promoters include the Tet system, (US Patents 5,464,758 and 5,814,618), the Ecdysone inducible system (No et al., Proc. Natl. Acad. Sci. (1996) 93 (8): 3346-3351; the T-RE$_x$™ system (Invitrogen Carlsbad, CA), LacSwitch® (Stratagene, (San Diego, CA) and the Cre-ER™ tamoxifen inducible recombinase system (Indra et al. Nuc. Acid. Res. (1999) 27 (22): 4324-4327; Nuc. Acid. Res. (2000) 28 (23): e99; US Patent No. 7,112,715; and Kramer & Fussenegger, Methods Mol. Biol. (2005) 308. 123-144) or any promoter known in the art suitable for expression in the desired cells.

The term "**protein of interest**" refers to any protein which may be useful for research, diagnostic or therapeutic purposes. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. Exemplary proteins of interest include, but are not limited to, molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin;

insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor, lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; members of the TNF and TNF receptor (TNFR) family, like tumor necrosis factor-alpha and -beta, CD40 ligand, Apo-2 ligand/TRAIL, DR4, DR5, DcR1, DcR2, DcR3, OPG, Fas ligand; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide, a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D, rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TG-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20, erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma, colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; thrombopoietin (TPO); interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope, gp120; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and variants and/or fragments of any of the above-listed polypeptides; as well as antibodies against various protein antigens like CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34, members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, an Apo-2L receptor such as Apo-2 (DR5), DR4, DcR1, DcR2, DcR3; and variants and/or fragments of the above-identified antibodies etc. In one embodiment of the invention, a protein of interest will comprise a protein which itself is capable

of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

The term "**production phase**" of the cell culture refers to the period of time during which cell growth has reached a plateau. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product

The term "**produced in a plant**" when used to describe the cell culture components disclosed herein. means that the protein has been obtained directly or indirectly from a plant cell. With respect to recombinant proteins, the term "produced in a plant" means that the recombinant protein has been expressed within a plant cell, and then purified from host cells proteins and lipids. Proteins that have been produced in a plant may be differentiated from proteins that have purified from other organisms by at least three criteria. 1) Proteins produced in a plant will typically comprise different patterns of post-translational modifications compared to proteins produced in different organisms, which may be readily identified by mass spectrum analysis 2) Proteins produced in a plant will typically comprise a different set of bound co-factors, lipids and contaminating proteins compared to the same protein produced in a different expression system, or purified from a different source. 3) Proteins produced in a plant may exhibit a different biological activity compared to the same protein produced or purified from a different source. The difference in activity in the protein produced in a plant may arise from a difference in the conformation, folding, aggregation state, post-translational modifications, or bound factors or co-purified contaminating proteins of the protein produced in a plant compared to a protein produced or isolated from a different organism. Differences in the specific activity of a protein produced in a plant may be readily established by routine assays using defined concentrations and assay conditions, for example, using cell growth, and factor synergism assays, as disclosed in Examples 11 to 19. Specifically a protein produced in a plant may exhibit at least at least 10 % greater cell growth activity either alone, or in combination with other factors compared to the same concentration of a non plant produced protein. Preferably, a protein produced in a plant may exhibit at least at least 20 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 30 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein. More preferably, a protein produced in a plant may exhibit at least at least 40 % greater cell growth activity either alone, or in combination with other factors,

compared to the same concentration of a non plant produced protein. Even more preferably, a protein produced in a plant may exhibit at least at least 50 % greater cell growth activity either alone, or in combination with other factors, compared to the same concentration of a non plant produced protein.

The terms "**isolated**" or "**purified**" when used to describe the cell culture components disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, the protein will be purified to at least 95% homogeneity as assessed by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step

The term "**recombinant protein**" or "**recombinant polypeptide**" refers to an exogenous, i.e., heterologous or foreign polypeptide, to the cells producing the polypeptide.

The term "**stress**" in the context of apoptosis or cell culture refers to non-optimal conditions for tissue culture including any combination of the following; the presence of toxins, nutrient or growth factor depletion or withdrawal, hypoxia, thermal stress (temperature is too high or too low compared to the preferred range), loss of cell-cell contacts, viral infection, osmotic stress (osmolality is too high or too low compared to the preferred range), oxidative stress, cell density (cell density is too high or too low compared to the preferred range), and pH stress (pH is too high or too low compared to the preferred range)

The term "**transformation**" refers to the transfer of one or more nucleic acid molecules into a host cell or organism. Methods of introducing nucleic acid molecules into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, scrape loading, ballistic introduction or infection with viruses or other infectious agents. "**Transformed**", "**transduced**", "**transgenic**", and "**recombinant**" refer to a host cell or organism into which a recombinant or heterologous nucleic acid molecule (e.g., one or more DNA constructs or RNA, or siRNA counterparts) has been introduced. The nucleic acid molecule can be stably expressed (i.e maintained in a functional form in the cell for longer than about three months) or non-stably maintained in a

functional form in the cell for less than three months i.e. is transiently expressed. For example, "transformed," "transformant," and "transgenic" cells have been through the transformation process and contain foreign nucleic acid. The term "untransformed" refers to cells that have not been through the transformation process.

The term "**transition phase**" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as pH, ion concentration, and temperature may shift from growth conditions to production conditions.

The term "**seed product**" includes, but is not limited to, seed fractions such as de-hulled whole seed, flour (seed that has been de-hulled by milling and ground into a powder) a seed extract, preferably a protein extract (where the protein fraction of the flour has been separated from the carbohydrate fraction), malt (including malt extract or malt syrup) and/or a purified protein fraction derived from the transgenic grain.

"**Seed maturation**" refers to the period starting with fertilization in which metabolizable reserves, e.g., sugars, oligosaccharides, starch, phenolics, amino acids, and proteins, are deposited, with and without vacuole targeting, to various tissues in the seed (grain), e.g., endosperm, testa, aleurone layer, and scutellar epithelium, leading to grain enlargement, grain filling, and ending with grain desiccation.

The term "**sequence similarity**" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin (see Reeck et al., supra). However, in common usage and in the instant application, the term "homologous", when modified with an adverb such as "highly", may refer to sequence similarity and may or may not relate to a common evolutionary origin.

In specific embodiments, two nucleic acid sequences are "substantially homologous" or "substantially similar" when at least about 85%, and more preferably at least about 90% or at least about 95% of the nucleotides match over a defined length of the nucleic acid sequences, as determined by a sequence comparison algorithm known such as BLAST, FASTA, DNA Strider, CLUSTAL, etc. An example of such a sequence is an allelic or species variant of the specific genes of the present invention. Sequences that are substantially homologous may also be identified by hybridization, e.g., in a Southern hybridization experiment under, e.g., stringent conditions as defined for that particular system.

Similarly, in particular embodiments of the invention, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acid residues are identical, or when greater than about 90% of the amino acid residues are similar (i.e., are functionally identical). Preferably the similar or homologous polypeptide sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Version 7, Madison, Wis.) pileup program, or using any of the programs and algorithms described above. The program may use the local homology algorithm of Smith and Waterman with the default values: Gap creation penalty = -(1+1/*k*), *k* being the gap extension number, Average match = 1, Average mismatch = -0.333.

As used herein and in the appended claims, the singular forms "a," "an," and "the," include plural referents unless the context clearly indicates otherwise. Thus, for example reference to "a molecule" includes one or more of such molecules, "a reagent" includes one or more of such different reagents, reference to "an antibody" includes one or more of such different antibodies, and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, miocrobiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. Practitioners are particularly directed to Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (Second Edition), Cold Spring Harbor Press, Plainview, N.Y.; Ausubel FM et al. (1993); *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; and Gelvin and Schilperoot, eds (1997) *Plant Molecular Biology Manual*, Kluwer Academic Publishers, The Netherlands;. DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990. In Situ Hybridization: Principles and Practice; Oxford University Press. M. J. Gait (Editor), 1984, Oligonucleotide Synthesis. A Practical Approach, Irl Press, D. M. J. Lilley and J. E. Dahlberg, 1992, Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA Methods in Enzymology, Academic Press; Using Antibodies: A Laboratory Manual: Portable Protocol NO. I by Edward Harlow, David Lane. Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7), Antibodies: A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-3,4-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, N.Y., Marcel

Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3 Each of these general texts is herein incorporated by reference.

The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods, compositions, reagents, cells, similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are described herein. All publications mentioned herein are incorporated herein, including all figures, graphs, equations, illustrations, and drawings, to describe and disclose specific information for which the reference was cited in connection with.

I. Methods of using the supplements of the invention

Frequently, cell culture media is supplemented with serum at concentration of 5 to 10%. Serum is a complex mixture containing undefined essential matter for cell growth. Commonly used serum is fetal calf serum (FCS) and fetal bovine serum (FBS). Due to its complex nature, lack of consistency, and the potential risk for pathogen contamination, cell culture industries and regulatory agencies are seeking alternatives to adding serum to cell culture media.

Cell culture components and cell culture supplements added to basal media serve distinct functions in promoting cell growth. These protein components are typically animal-derived. With the increase in the concern of potential pathogen contamination from animal sourced material, cell culture industries are trying to develop animal-component-free media. While some successes have been made with the use of plant-based hydrolysates and recombinant proteins produced using microbial systems, plant-based hydrolysates are an undefined mixture of components and recombinant protein from microbial sources are too expensive to use in routine cell culture An alternative source of non-animal-derived components would be beneficial for industries that depend on cell culture techniques.

Accordingly in one embodiment the instant invention provides a method for culturing cells,

including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

Accordingly in one embodiment the instant invention provides a method for culturing cells, including providing a cell culture media supplemented with two or more proteins selected from the group consisting of lactoferrin, transferrin, albumin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof; and introducing cells to be cultured into the cell culture media. The cells grown in the cell culture media exhibit improved growth characteristics as compared to cells grown in an unfortified cell culture media.

According to further embodiments, the combination of proteins that is used in the methods of culturing cells and supplements of the invention provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, the supplements comprising one combination of proteins that may be used in the methods of culturing cells includes lactoferrin and albumin.

In another aspect, the supplements comprises a combination of transferrin related proteins and albumin which provide a synergistic effect on cell growth and productivity.

A further aspect of the invention includes methods for achieving an improved growth rate of cultured cells, and higher productivity of the cultured cells, adding a supplement of the present invention to the cell culture medium.

The claimed supplements are useful in a wide range of applications for tissue and cell culture and recombinant protein production where they provide for significant improvements in preventing apoptosis and improving cell viability during tissue culture, and in particular in response to stress

Apoptosis involves a series of biochemical events leading to a characteristic cell morphology and death. These changes include, changes to the cell membrane such as loss of membrane asymmetry and attachment, cellular blebbing cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (extrinsic inducers) or intracellularly (intrinsic inducers). Extracellular signals may include toxins, hormones, growth factors, nitric oxide, cytokines, which may be present to different degrees in tissue culture media. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis, and thus influence overall cell viability A number of intracellular components, including ATP content, calcium level, and a number of apoptotic and anti-apoptotic genes also help regulate apoptosis. A cell may initiate intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. Stress inducing agents encountered during tissue culture include for example toxins, associated with tissue culture components such as endotoxins, and heavy metals that leach from plastic ware, transfection reagents (e.g Lipofectamine and similar lipid based transfection reagents), viral transformation, nutrient and growth factor deprivation, associated with serum free culture, or cell differentiation protocols hypoxia and oxidative stress associated with high density culture in a bioreactor and increased intracellular calcium concentration, for example, by damage to the membrane caused by detergents and electroporation.

Before the actual process of cell death occurs, the apoptotic signals must overcome regulatory proteins which act as gatekeepers overseeing the activation of the apoptosis pathway. In vivo, this step allows the process to be stopped, should the cell no longer need to die. Several proteins are involved at this step, though two main mechanisms of regulation have been identified and include those associated with mitochondria functionality, and those directly involved in transducing the signal via adaptor proteins to the apoptotic mechanisms.

Cells grown under cell culture conditions may experience cellular stresses associated with routine tissue culture procedures, as described above which may trigger apoptotic signals and increase the susceptibility of the cells to apoptosis. For example, nutrient deprivation associated with serum free culture, oxidative stress associated with high density growth in a bioreactor, the use of cytotoxic compounds associated with DNA transfection reagents, and thermal stresses associated with cryopreservation, may predispose the cell to enter apoptosis. By enhancing the ability of a cell to survive such signals it is possible to improve cell viability

during these procedures. by preventing the cells commitment to cell death, thereby improving the success and utility of these approaches.

Recently a number of genes in eukaryotic cells have been identified which inhibit the onset or reduce the effects of apoptosis. Some of these genes inhibit caspase dependent apoptotic pathways in the cell, and in fact transfecting cells with anti-apoptotic genes may be useful in prolonging the life and productivity of transfected cells grown under biologically demanding conditions (US Patent Nos., 6,586,206; 7,531,327, US Patent Application US 2009/0170165; US2009/0181426).

Additionally the addition of exogenous heat shock proteins has in some cases been shown to improve the survival of cells in culture under a variety of conditions. (Novoselova et al., J. Neurochem. 94 597-606 (2005); Tidwell et al., Cell Stress & Chap 9 (1) 88-90 (2004); Guzhova et al., Cell Stress & Chap. 3 (1) 67-77 (1998); Hounenou et al., Cell Stress & Chap 1 (3) 161-166 (1996); Johnson et al , In vitro Cell. Dev. Biol., 29A 807-812 (1993)

The present invention is based in part on the demonstration that plant derived recombinant cell culture component proteins surprisingly enhanced the cell growth and viability when added in specific mixtures to mammalian cells grown in culture. Surprisingly some combinations of proteins provide for a synergistic effect on cell growth and productivity which provides unexpected benefits compared to the use of the individual proteins Specifically, such supplements comprising plant derived recombinant albumin and transferrin related proteins result in improved culture viability, extended cell survival, improved rates of cell growth and improved yields of recombinant proteins produced from tissue culture bioreactors. Because the supplements show unexpectedly improved activity and stability they offer significant improvements compared to the use of standard recombinant or purified proteins.

The supplements disclosed in the present application are useful, for example, for improving cell viability and in accelerating the rate of cell growth of cells grown in culture. In one aspect, the supplements of the invention are useful for improving or enhancing the yield of the recombinant proteins from the cell cultures. Further improvements provided by the invention are described in detail below.

In one embodiment, the present invention includes a method for enhancing cell growth of a cell in culture comprising the addition of a supplement to the cell culture medium

In one embodiment. the present invention includes a method for enhancing the productivity of a cell that has been adapted to serum free media comprising the addition of a supplement to the serum free media

In one embodiment, the present invention includes a method for reducing the accumulation of lactate in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method or reducing the consumption of glucose and other sugars in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment. the present invention includes a method of reducing time required to produce protein from start of culture to harvest in a bioreactor comprising the addition of a supplement to cells in culture in the bioreactor.

In one embodiment, the present invention includes a method for improving the viability of cells in a bioreactor comprising the addition of a supplement to the bioreactor

In one embodiment, the present invention includes a method for improving the viability of cells grown under serum free conditions comprising the addition of a supplement to the serum free medium.

In one embodiment, the present invention includes a method for improving the viability of cells when plated at low density comprising the addition of a supplement to the cell culture medium

In one embodiment, the present invention includes a method for improving the viability of cells grown from single cell clones comprising the addition of a supplement to the cell culture medium.

In one embodiment, the present invention includes a method for improving the viability of primary cells grown in culture comprising the addition of a supplement to the culture medium.

In one embodiment, the present invention includes a method for improving the viability of cells after transfection comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after transfection

In one embodiment, the present invention includes a method for improving the viability of cell after cryopreservation comprising the addition of a supplement to the cell culture medium prior to, during, or immediately after cryopreservation or thawing.

In one embodiment, the present invention includes a method for improving the rate of cell growth or viability of stem cells grown in culture comprising the addition of a supplement of the present invention to the cell culture media.

In one embodiment, the present invention includes a method for improving the yield of a recombinant product produced from cells in culture comprising the addition of a supplement of the present invention to the cell culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the purification of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for reducing the proteolysis of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for improving the bioactivity of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the stability of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In one embodiment, the present invention includes a method for improving the assembly of a recombinant product produced from cells in culture, comprising the addition of a supplement to

the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a more human pattern of glycosylation of a recombinant product produced from cells in culture, comprising the addition of a supplement to the culture media during one or more of the growth phase, transition phase, or production phase of the culture.

In one embodiment, the present invention includes a method for creating a recombinant product produced from cells in culture with less immunogenicity, comprising the addition of a supplement comprising recombinant albumin to the culture media during one or more of the growth phase, transition phase, or production phase of the culture

In any of these methods, the supplements of the invention, by increasing host cell viability in culture (and during fermentation), provide for a simple and cost effective method to increase the yield, and or purity, bioactivity, stability and assembly of functional recombinant protein Additionally, the supplements of the invention, by decreasing or inhibiting apoptosis in the cell culture, can decrease the number or presence of adverse proteases in the culture media and protect the expressed protein of interest against proteolytic degradation, thereby increasing the quality of the protein of interest produced, as evidenced by increased amounts of active protein, and increased yields of intact protein. Additionally Applicants have found that the supplements of the invention may protect the cells against potential adverse effects of agents like detergents, heavy metals and endotoxin contaminates present in the culture components, or protect the cells from toxic reagents introduced to the cells during transfection or cryopreservation.

In any of the claimed methods, the supplements of the invention can be added directly, or admixed, to the culture media at any convenient time, for example when changing the media, passaging the cells, or when plating out the cells at low density. Optionally, the supplement is added to the culture media at the beginning (at the time of initiating, day 0) of the cell culturing process. In one aspect the supplements of the invention may be added before an anticipated stressful event, for example before cryopreservation, transfection or serum withdrawal, etc.

In another aspect, the supplement is added to the culture media during the culturing of the cells prior to the point when induction of typically apoptosis occurs For example, during a large scale cell culture, induction of apoptosis can be observed on about day 3 or day 4 of the culture, and therefore, the supplement will preferably be added prior to day 3 or day 4. Optionally, a

desired quantity of the supplement is added throughout, or for the duration of, the cell culture, for instance, on a daily basis for the entire fermentation. As an example, for a 5 day culture, the supplement could be added at day 0, and every 24 hours thereafter until the culture is terminated.

Accordingly in one embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a bioreactor by adding a supplement of the invention to the bioreactor. In one embodiment, the bioreactor comprises bacterial cells. In another aspect the bioreactor comprises yeast cells. In another aspect the bioreactor comprises plant cells. In another aspect the bioreactor comprises mammalian cells

In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in bacterial cells, by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in yeast cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in a plant cells by adding the supplement of the invention to the cell culture. In another embodiment, the invention provides a method of improving the yield and quality of a recombinant protein produced in insect cells by adding the supplement of the invention to the cell culture. In another embodiment the invention provides a method of improving the yield and quality of a recombinant protein produced in mammalian cells by adding the supplement of the invention to the cell culture.

In another embodiment, the invention provides a method to increase the yield of the production phase of a cell culture system and thereby increase the productivity of a bioreactor by adding the supplement of the invention to the cell culture system prior to, or during the production phase of the cell culture system.

In one aspect of this method the yield of the production phase is increased by about 10%. In one aspect of this method the yield of the production phase is increased by about 20%. In one aspect of this method the yield of the production phase is increased by about 30%. In one aspect of this method the yield of the production phase is increased by about 40%. In one aspect of this method the yield of the production phase is increased by about 50%. In one aspect of this method the yield of the production phase is increased by about 60%. In one aspect of this method the yield of the production phase is increased by about 70%. In one aspect of this method the yield of the production phase is increased by about 80%. In one

aspect of this method the yield of the production phase is increased by about 90%. In one aspect of this method the yield of the production phase is increased by about 100%. In one aspect of this method the yield of the production phase is increased by about 200%. In one aspect of this method the yield of the production phase is increased by about 500%.

In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which the supplement was not added. In one aspect of any of these methods, the yield is increased compared to a control cell culture system to which is added a supplement lacking either a transferrin related protein, or recombinant serum albumin. In one aspect, the yield is increased compared to the summed effect of the recombinant albumin and the transferrin related protein when added alone when measured under the same culture conditions

In another embodiment, the invention provides a method to produce a protein of interest at a temperature that is elevated compared to normal growth conditions for the production of that protein, comprising the addition of a supplement of the invention to cells expressing the protein of interest.

In another embodiment, the invention provides a method to decrease the amount of aggregates formed in a cell culture expression system by aggregate prone proteins of interest comprising the addition of a supplement of the invention to the cell culture expression system, whereby the aggregation state of the protein is reduced.

In another embodiment, the invention provides a method to increase the activity of a protein of interest protein expressed by a cell by preventing the denaturation and aggregation of the recombinant protein comprising the addition of a supplement of the invention to the cell, whereby the specific activity of the protein of interest is increased.

In another embodiment, the invention provides a method to improve the expression of proteins in a cell culture expression system that are aggregation prone, cause precipitation to occur, or are toxic themselves to the cells comprising the addition of a supplement of the invention to the cell culture expression system, whereby the expression of the protein of interest is increased.

The amount of supplement to add in any of these methods will depend on various factors, for instance, the type of host cell, the cell density, protein of interest and culture conditions, etc Determining the desired concentration of supplement to be added to the culture media is within the skill in the art and can be ascertained empirically by routine optimization and without undue experimentation.

The skilled artisan will readily appreciate that different cell types will have different magnitudes of responses to the supplement of the invention, and this will be determined, to some degree, by the amount or type of the proteins in the supplement. Additionally different densities of cells will require appropriate adjustment in the total amount of supplement as well as the concentration of the individual proteins added to the culture to account for the increased cell number. Additionally cells grown in suspension culture or via adherent culture will have different membrane surface areas available for protein entry and will typically exhibit different rates and degrees of response. Therefore, one should choose a concentration which provides for a sufficient inhibition of apoptosis, or increase in viability, or net cell growth. Typically the supplements of the invention will be added to a final concentration of about 0.1 %, about 0.5 %, about 1%, about 2%, about 3 %, about 4 %, about 5 %, about 6%, about 7%, about 8%, about 9%, about 10 %, about 11 %, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, about 20%, about 25%, about 30%, about 40%, or about 50% wt/wt, or wt /volume

There will typicelly be an upper range of concentration of the supplement beyond which further increases in cell survival do not occur. As described in the Examples below, Applicants have found that the supplements of the invention can inhibit apoptosis when added to cell cultures at a concentration of about 200 mg /L to about 2g/L, or more preferably about 200 mg/L to about 1000 mg/L, or more preferable about 250 to about 500 mg/L.

II. Supplements

The cell culture media, methods for producing supplemented cell culture media, and methods of culturing cells using fortified cell culture media in accordance with the present invention may include components derived from animal, plant, bacterial, yeast, and insect sources. According to one aspect of the invention, the components are derived from plant sources, and preferably from monocot plants. The components may be combined with inorganic salts such as NaCl, KCl, NaH_2PO_4, $NaHCO_3$, $CaCl_2$, and $MgCl_2$ and other ingredients such as amino acids, vitamins, growth factors, sugars, and antibiotics to form a variety of different cell culture media. According to further embodiments, the proteins contained in the cell culture media are recombinant proteins, preferably recombinant human proteins. According to still further embodiments, one or more of the proteins in the cell culture media are plant-produced heterologous proteins, preferably two or more of the proteins in the cell culture media are plant-produced heterologous proteins, and more preferably all of the proteins in the cell culture media

are plant-produced heterologous proteins.

According to another aspect, one of the proteins is albumin, and at least one additional protein is selected from the group consisting of growth factors, lactoferrin transferrin, insulin, growth hormone, fibronectin attachment factor, lamin attachment factor, collagenase, platelet derived growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor, thymic factors, haptocorin, lactahedrin, lactoperoxidase, alpha-fetoprotein, immunoglobin, alpha-lactalbumin, and derivatives, fractions, and biologically-active fragments thereof

According to further embodiments, the combination of proteins that is used in the methods of producing the supplemented cell culture media provides a synergistic effect on the growth and/or productivity of the cells present in the cell culture media. According to still further embodiments, one combination of proteins that may be used in the methods of producing the cell culture media includes a combination of albumin with at least one other protein selected from the group consisting of lactoferrin, transferrin, melanotransferrin, and ovotransferin.

In one aspect the supplements of the invention comprise plant derived recombinant human serum albumin.

The term **"albumin"**, refers to all naturally-occurring and synthetic forms of albumin. Preferably, the term "albumin" refers to recombinant albumin. In one aspect the albumin is from a vertebrate. In one aspect the albumin is from a mammal. In a further embodiment the albumin is human. In another aspect, the recombinant albumin is produced from a plant cell. In one particularly preferred embodiment the recombinant albumin is produced from transgenic rice (Oryza sativa) Representative species and Gene bank accession numbers for various species of albumin are listed below in **Table D 1.**

Table D1

Exemplary Albumin genes

Species	Gene Bank Accession number
Human	NP_000468.1
Pan troglodytes	XP_517233.2
Canis lupus familiaris	XP_855557.1
Bos taurus	NP_851335.1
Mus musculus	NP_033784.1

Rattus norvegicus	NP_599153.1
Gallus gallus	NP_990592.1

It will be understood that for the recombinant production of albumin in different species it will typically be necessary to codon optimize the nucleic acid sequence of the gene for the host organism in question. Such codon optimization can be completed by standard analysis of the preferred codon usage for the host organism in question, and the synthesis of an optimized nucleic acid via standard DNA synthesis. A number of companies provide such services on a fee for services basis and include for example, DNA2.0, (CA, USA) and Operon Technologies, (CA, USA).

The albumin may be in its native form, i.e., as different allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the albumin, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the albumin.

Fragments of native or synthetic albumin sequences may also have the desirable functional properties of the peptide from which they derived and may be used in any of the methods of the invention The term "fragment" as used herein thus includes fragments of albumin provided that the fragment retains the biological or therapeutically beneficial activity of the whole molecule

For example, albumin contains at least 2 high affinity multi- metal binding sites for a number of physiologically important metals ions including copper, zinc cadmium and nickel. (Carter et al , Advances in Protein Chemistry 45 153-203 (1994), Bal et al., J. Inorg Biochem 70 (1) 33-39 (1998), Blindauer et al., J. Biol. Chem. 284 (34) 23116-24 (2009); US Patent No. 6,787,636). Since trace amounts of these metals are typically present in the recombinant production of albumin, a significant amount of these metal ions can be become chelated to the protein. The binding of these ions, and in particular the binding of cadmium and nickel to recombinant albumin is associated with cellular toxicity of the protein when added to cells as a tissue culture component

Accordingly, in one aspect, the albumin of the present invention can comprise a fragment of albumin that includes the deletion of one or amino acids involved in the multi-metal binding sites of albumin. In one aspect the albumin fragment is created by the deletion of one or more amino acids at the N-terminus of the mature protein. In another aspect the albumin can comprise one or more deletions or mutations of any of the amino acids involved in the N-terminal metal binding site of albumin. In one aspect, the amino acids to be deleted or mutated are independently selected from the first 10 amino acids of the mature protein.

The term "derivative" as used herein thus refers to albumin sequences or fragments thereof, which have modifications as compared to the native sequence. Such modifications may be one or more amino acid deletions, additions, insertions and/or substitutions. These may be contiguous or non-contiguous. Representative variants may include those having 1 to 20, or more preferably 1 to 15, 1 to 10, or 1 to 5 amino acid substitutions, insertions, and / or deletions as compared to any of genes listed in Tables D1. The substituted amino acid may be any amino acid, particularly one of the well-known 20 conventional amino acids (Ala (A); Cys (C); Asp (D); Glu (E); Phe (F), Gly (G); His (H); Ile (I); Lys (K); Leu (L); Met (M); Asn (N); Pro (P); Gln (Q); Arg (R); Ser (S); Thr (T); Val (V); Trp (W), and Tyr (Y)). Any such variant or derivative of albumin may be used in any of the methods of the invention.

Accordingly, the albumin of the invention can comprise amino acid deletions, insertions or mutations in any of the functional binding domains of albumin. In one aspect the albumin may comprise a mutation in a binding domain of albumin. In one aspect the mutated binding domain is a domain involved in the binding of aspirin, warfarin, diazepam, digitoxin, diofibrate, ibuprofen or AZT, as outlined is US Patent No. 5,780,593, or a multimetal binding site as outlined in Blindauer et al., J. Biol Chem. 284 (34) 23116-24 (2009)

Thus, the albumin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native albumin amino acid sequences, for example, to any of the native albumin gene sequences listed in **Table D1**. Alternatively, the albumin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with albumin listed in **Table D1**. In a preferred embodiment, the albumin for use in any of the methods of the present invention is at least 80% identical to the mature secreted human serum albumin (**SEQ. ID. No. 1**) as shown underlined in the below (Swiss-Prot P02768).

MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF

EIHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP
ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF
FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV
ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR
RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE
QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV
LNQLCVLHEK TPVSDRVTKC CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL
SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
AASQAALGL

Fusion proteins of albumin to other proteins are also included, and these fusion proteins may enhance, activity, targeting, stability or potency.

Chemical modifications of the native albumin structure which retain or stabilize albumin activity or biological half-life may also be used with any of the methods described herein. Such chemical modification strategies include without limitation pegylation, glycosylation, and acylation (see Clark et al.: *J. Biol. Chem.* 271(36): 21969-21977, 1996; Roberts et al., *Adv. Drug. Deliv. Rev.* 54(4): 459-476, (2002), Felix et al., *Int J. Pept. Protein. Res.* 46(3-4): 253-264, (1995); Garber Diabetes Obes. Metab 7 (6) 666-74 (2005)) C- and N-terminal protecting groups and peptomimetic units may also be included.

Isomers of the native L-amino acids, e.g., D-amino acids may be incorporated in any of the above forms of albumin, and used in any of the methods of the invention. All such variants, derivatives, fusion proteins, or fragments of albumin are included, may be used in any of the methods claims or disclosed herein, and are subsumed under the term "albumin"

In one aspect of the supplements of the invention the albumin is recombinant human serum albumin that has been produced in a plant. In another aspect, the albumin comprises less than about 2% aggregated albumin. In another aspect the albumin comprises less than about 1 % aggregated albumin.

In one embodiment, the supplements of the invention comprise preparations of the co-purified recombinant albumin and rice hsps that are also essentially free of detergents and endotoxins which would otherwise mask or inhibit the positive impact of the hsp. In one aspect the supplements of the invention have less than about 1 EU of endotoxin, and the albumin is at least about 95 % pure. In another aspect the supplements of the invention comprise a

composition comprising recombinant albumin bound to a heat shock protein. In another aspect the supplements of the invention comprise recombinant albumin and a rice hsp70 homolog. In one aspect the rice hsp70 homolog is selected from HSP70, Bip and rice stromal protein

The terms "**heat shock protein**", "**HSP**" or "**hsp**", as used herein includes all naturally-occurring and synthetic forms of the heat shock protein super family that retain anti-apoptotic activity. Such heat shock proteins include the small heat shock proteins/HSPB family. Hsp40/DnaJ family, HSP70/ HSPA family, HSP90 / HSPC family, HSP110/ HSPH family and chapererone family, as well as peptide fragments and protein complexes of two or more heat shock proteins or nucleotide exchange factors (for example, complexes of HSP70 & HSP40) derived therefrom. The heat shock proteins may be in their native form, i.e., as different variants as they appear in nature in different species which may be viewed as functionally equivalent variants, or they may be functionally equivalent natural derivatives thereof, which may differ in their amino acid sequence, for example, by truncation (e g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical derivatives, including post-translational modifications and degradation products of the HSPs, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, oxidatized, isomenzed, and deaminated variants of the HSP.

The term "**transferrin related protein**" refers to transferrin family proteins including transferrin, lactoferrin, melanotransferrin, and ovotransferin. The crystal structures of transferrin family members across all species that have been solved to date show a high degree of structural similarity, which is not surprising since transferrin, lactoferrin and ovotransferrin share 60-80% sequence identity. (Wally and Buchanan, Biometals (2007) 20 249-262). All transferrin related proteins contain two domains of around 340 residues, which are thought to have evolved from an ancient duplication event. For serum transferrin, ovotransferrin and lactoferrin each of the duplicated lobes binds one atom of Fe (III) and one carbonate anion. With a few notable exceptions each iron atom is coordinated to four conserved amino acid residues, an aspartic acid, two tyrosines, and a histidine, while anion binding is associated with an arginine and a threonine in close proximity. (Anderson et al., (1989) J. Mol. Biol. 209 711-734).
Human transferrin and lactoferrin share over 61 % sequence identity and have similar three dimensional structures. Transferrins transport iron in the blood by picking up free ferric iron and delivering it to cells in a receptor-mediated endocytotic process in which the TF-receptor complex is internalized, iron is released in the endosome, and the complex is recycled to the cell surface where the TF is released. In contrast, no iron transport role for lactoferrin or

ovotransferrin is known. By comparison, these proteins are thought to function to prevent invading bacteria from acquiring iron, by sequestering all available iron. Human transferrin releases iron at a higher pH than does human lactoferrin which likely relates to the need for human transferrin to release iron after endocytosis into the endosome, and the need for lactoferrin to hold onto iron in low pH environments such as the stomach (Baker & Backer (2004) Biometals 17 209-216).

The term **"transferrin"** refers to all naturally-occurring and synthetic forms of transferrin. In one aspect, the term "transferrin" refers to recombinant transferrin. In one aspect, the term "transferrin" refers to plasma derived transferrin. In one aspect the transferrin is from a vertebrate. In one aspect the transferrin is from a mammal. In a further embodiment the transferrin is human. In another aspect the recombinant transferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant transferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of transferrin are listed below in **Table D2**.

Table D2

Exemplary Transferrin genes

Species	Gene Bank Accession number
Homo sapiens	NP_001054.1
Canis lupus familiaris	XP_864550.1
Bos taurus	NP_803450.2
Mus musculus	NP_598738.1
Rattus norvegicus	NP_001013128.1
Gallus gallus	NP_990635.1
Danio rerio	NP_001015057.1

The transferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of the transferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of the transferrin.

The transferrin which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native transferrin amino acid sequences, for example, to any of the native transferrin gene sequences listed in **Table D2**. Alternatively, the transferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with transferrin listed in **Table D2**. In a preferred embodiment, the transferrin for use in any of the methods of the present invention is at least 80% identical to the mature human transferrin.

The term **"Lactoferrin"** refers to all naturally-occurring and synthetic forms of Lactoferrin. In one aspect, the term "Lactoferrin" refers to recombinant Lactoferrin. In one aspect, the term "Lactoferrin" refers to milk derived Lactoferrin. In one aspect the Lactoferrin is from a vertebrate. In one aspect the Lactoferrin is from a mammal. In a further embodiment the Lactoferrin is human. In another aspect the recombinant Lactoferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant Lactoferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of Lactoferrin are listed below in **Table D3.**

Table D3

Exemplary Lactoferrin genes

Species	Gene Bank Accession number
Homo sapiens	AAA59511.1
Sus scrofa	AAA31059.1
Camelus dromedarius	CAB53387.1
Bos taurus	AAA30610.1
Equus caballus	CAA09407.1

The Lactoferrin may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions, insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of Lactoferrin are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of Lactoferrin. Lactoferrin which may be used in any of the methods of the

invention may have amino acid sequences which are substantially homologous, or substantially similar to the native Lactoferrin amino acid sequences, for example, to any of the native Lactoferrin gene sequences listed in **Table D3**. Alternatively, the Lactoferrin may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with Lactoferrin listed in **Table D3**. In a preferred embodiment, the Lactoferrin for use in any of the methods of the present invention is at least 80% identical to the mature human Lactoferrin.

The term "**melanotransferrin**" refers to all naturally-occurring and synthetic forms of melanotransferrin. In one aspect, the term "melanotransferrin" refers to recombinant melanotransferrin In one aspect, the term "melanotransferrin" refers to melanotransferrin purified from a cell or tissue. In one aspect the melanotransferrin is from a vertebrate. In one aspect the melanotransferrin is from a mammal. In a further embodiment the melanotransferrin is human. In another aspect the recombinant melanotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant melanotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of melanotransferrin are listed below in **Table D4.**

Table D4

Exemplary **melanotransferrin** genes

Species	Gene Bank Accession number
Homo sapiens	AAA59992.1
Oryctolagus cuniculus	NP_001075461.1
Mus musculus	NP_038928.1
Canis lupus familiaris	XP_545158.2
Gallus gallus	CAA63003.1

The term "ovotransferrin" refers to all naturally-occurring and synthetic forms of ovotransferin. In one aspect, the term "ovotransferrin" refers to recombinant ovotransferrin In one aspect the ovotransferin is from a vertebrate. In one aspect the ovotransferin is from an avian. In another aspect the recombinant ovotransferrin is produced from a plant cell. In one particularly preferred embodiment the recombinant ovotransferrin is produced from transgenic rice (Oryza sativa). Representative species and Gene bank accession numbers for various species of ovotransferrin include CAA26040.1

The term "**insulin related growth factor**" **or** "IGF-1" refers to all naturally-occurring and

synthetic forms of insulin related growth factor, including IGF-1, IGF-1A, IGF-1B, IGF-2A, and IGF-2B. In one aspect, the term "insulin related growth factor" refers to recombinant insulin related growth factor. In one aspect, the term "insulin related growth factor" refers to insulin related growth factor purified from a cell or tissue In one aspect the insulin related growth factor is from a vertebrate. In one aspect the insulin related growth factor is from a mammal. In a further embodiment the insulin related growth factor is human. In another aspect the recombinant insulin related growth factor is produced from a plant cell. In one particularly preferred embodiment the recombinant insulin related growth factor is produced from transgenic rice (Oryza sativa) Representative species and Gene bank accession numbers for various species of insulin related growth factor are listed below in **Table D5**.

Table D5
Exemplary insulin related growth factor genes

Species	Gene Bank Accession number
Homo sapiens	P01343 P05019 CAA01954.1 CAA40093.1 CAA40092.1
Bos taurus	NP_001071296
Mus musculus	NP_001104746.1 NP_034642.2 NP_908941.1 NP_001104745.1 NP_001104744.1
Xenopus laevis (African clawed frog)	Q90WW4
Danio rerio (zebrafish	NP_571900

The **insulin related growth factor** may be in its native form, i.e., as different apo forms, or allelic variants as they appear in nature, which may differ in their amino acid sequence, for example, by truncation (e.g., from the N- or C-terminus or both) or other amino acid deletions, additions insertions, substitutions, or post-translational modifications. Naturally-occurring chemical modifications including post-translational modifications and degradation products of

insulin related growth factor, are also specifically included in any of the methods of the invention including for example, pyroglutamyl, iso-aspartyl, proteolytic, phosphorylated, glycosylated, reduced, oxidatized, isomerized, and deaminated variants of **insulin related growth factor**. **Insulin related growth factor** which may be used in any of the methods of the invention may have amino acid sequences which are substantially homologous, or substantially similar to the native **insulin related growth factor** amino acid sequences, for example, to any of the native **insulin related growth factor** gene sequences listed in **Table D5**. Alternatively, the **insulin related growth factor** may have an amino acid sequence having at least 30% preferably at least 40, 50, 60, 70, 75, 80, 85, 90, 95, 98, or 99% identity with **insulin related growth factor** listed in **Table D5**. In a preferred embodiment, the **insulin related growth factor** for use in any of the methods of the present invention is at least 80% identical to the mature human **insulin related growth factor**.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 5, about 1 to 10, about 1 to 15, about 1 to 20 or about 1 to 25. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 0.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 1 about 1 to 2, about 1 to 3, about 1 to 4 or about 1 to 5. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.5 to 0.8 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.5 about 1 to 0.75, about 1 to 1.0 about 1 to 1.25, about 1 to 1.5, about 1 to 1.75 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.8 to 1.5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.25 about 1 to 0 33, about 1 to 0.66 about 1 to 1 00, about 1 to 1 33, about 1 to 1 66 or about 1 to 2.0. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 1.0 to about 1 5 g/L. In one aspect the supplement exhibits at least a 30% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of lactoferrin and recombinant albumin mixed in a ratio of lactoferrin to albumin of about 1 to 0.8, about 1 to 1 or about 1 to 1.2. In one aspect of any of these supplements, the concentration of lactoferrin in the culture is about 0.1 to about 1.5 g/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 20, about 1 to 100. or about 1 to 200. In one aspect of any of these supplements the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 500, about 1 to 1000, or about 1 to 2000. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and recombinant albumin mixed in a ratio of transferrin to albumin of about 1 to 3000. about 1 to 4000, or about 1 to 5000.

In one aspect of any of these supplements, the concentration of transferrin in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of transferrin and albumin mixed in a ratio of transferrin to albumin of about 1 to 80, about 1 to 100 or about 1 to 120. In one aspect of any of these supplements, the concentration of transferrin in the culture is about 3 to about 8 u g/L. In one aspect the supplement exhibits at least an 80% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In one aspect the supplements comprise a mixture of IGF-1 and recombinant albumin mixed in a ratio of IGF-1 to albumin of about 1 to 100000, about 1 to 10000, or about 1 to 1000 In one aspect of any of these supplements, the concentration of IGF-1 in the culture is about 1 to about 10 ug/L. In one aspect the supplement exhibits at least a 50% increase in productivity compared to the additive effect of each cell culture component alone when added to the culture at the same concentration and measured under the same culture conditions as the combination of tissue culture components.

In another aspect of the invention, the supplements contain one or more additional factors selected from the group consisting of a growth factor, sodium selenate and ethanolamine

In another aspect of the invention, the growth factors are independently selected from insulin, Epidermal Growth Factor (EGF), Fibroblast Growth Factors 1-23 (FGF), Insulin-like Growth Factor-1(IGF), keratinocyte growth factors 1 & 2(KGF), and Leukemia Inhibitory Factor (LIF). In one aspect the growth factor is insulin. In another aspect the growth factor is transferrin.

In one aspect, the supplements of the invention may be prepared by mixing the isolated cell culture components in aqueous solution.

Liquids of known concentration can also be combined containing one component part A (albumin or another cell culture component), to a liquid containing part B (such as a transferrin related protein or IGF-1) to obtain a ratio that contains the desired ratios of the components

Powdered, lyophilized, or otherwise dried powder (albumin) can be added directly to an aqueous solution containing the cell culture component (such as transferrin related protein) in order to obtain a ratio based on dry weight of albumin at any desired range with respect to the other cell culture components Powdered, lyophilized, or otherwise dried albumin can also be blended with the cell culture component powder on a mass to mass basis to obtain a ratio that is completely based on gravimetrics. The resulting powder can be dissolved at concentrations ranging from very low (picomolar) to very high concentrations (millimolar) in suitable buffers that are common to the art to reconstitute the cell culture components

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin and one or more transferrin related proteins and /or IGF-1. Such supplements will commonly be prepared as sterile liquid or powder form The total amount of transferrin related protein in the composition may vary from 50% to about 0.0001% of weight of the cell culture component. In other aspects the amount of transferrin related protein in the composition may vary from about 0.01% to about 0.02%, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0 02% to about 0.06%, or about 0.02% to about 0.08%. In another aspect the amount of transferrin related protein in the composition is greater than about 0 01%, or more preferably greater than about 0.05%, or more preferably greater than about 0.1% wt /wt, or more preferably greater than about 0.2% wt/wt transferrin related protein with respect to albumin.

In one aspect of any of the claimed supplements, the recombinant albumin is essentially free of endotoxin and detergents. In another aspect the albumin has less than about 1 EU /mg of endotoxin. In yet another aspect, the albumin contains less than about 10 ppm detergent. In another aspect of any of the claimed supplements, the albumin has a purity of greater than 95 %.

In another of any of the claimed supplements, the supplement comprises recombinant albumin which is bound to a rice heat shock protein, wherein the complex has less than about 1 EU of endotoxin and is at least 95 % pure with respect to the albumin. In one aspect the recombinant albumin is produced in rice.

In another aspect of any of these methods the supplement contains recombinant albumin as the cell culture component, and the albumin is essentially free of aggregated albumin In another aspect of any of these supplements the recombinant albumin has less than about 2 % aggregated albumin.

In one aspect, supplements of the present invention will accordingly comprise recombinant albumin with one or more bound hsps. Such supplements will commonly be prepared as sterile liquid or powder form. The total amount of hsp in the albumin composition may vary from 5 % to about 0.001% of weight of albumin. In other aspects the amount of hsp in the albumin composition may vary from about 0.01% to about 0.02 %, or about 0.01% to about 0.09%, or about 0.02% to about 0.04%, or about 0.02% to about 0.06%, or about 0.02% to about 0.08 %. In another aspect the amount of hsp in the albumin composition is greater than about 0 02%, or more preferably greater than about 0.03%, or more preferably greater than about 0.04% wt /wt, or more preferably greater than about 0.05% wt/wt hsp with respect to albumin

III. Production of Cell culture media components

Albumin and transferrin related proteins, as well as other cell culture media components for use in the supplements of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (see below), or by chemical synthesis, using, for instance, automated peptide synthesizers, or any combination of such methods. The means for preparing such polypeptides are well understood in the art.

For recombinant production, host cells can be genetically engineered to incorporate nucleic acids encoding the cell culture media component. Typically the nucleic acid will be codon optimized for high level expression in the expression system of choice, and incorporated into an expression vector to enable the expression of the protein of interest in the host cell. Vectors can exist as circular, double stranded DNA, and range in size form a few kilobases (kb) to hundreds of kb Preferred cloning vectors have been modified from naturally occurring plasmids to facilitate the cloning and recombinant manipulation of polynucleotide sequences. Many such vectors are well known in the art and commercially available; see for example, by Sambrook (In. "Molecular Cloning: A Laboratory Manual," second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, (1989)), Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608(1980).

General and specific techniques for producing proteins from plant cells may be obtained from the following patents and applications, each of which is incorporated herein in its entirety by reference: U.S. Pat Pub. No. 2003/0172403 A1 ("Plant Transcription Factors and Enhanced Gene Expression"); U.S. Pat. No. 6,991,824 ("Expression of Human Milk Proteins in Transgenic

Plants"); U.S. Pat. Pub. No. 2003/0221223 ("Human Blood Proteins Expressed in Monocot Seeds"); U.S. Pat. Pub. No. 2004-0078851 ("Production of Human Growth Factors in Monocot Seeds"); U S. Pat. Pub. No. 2004/0063617 ("Method of Making an Anti-infective Composition for Treating Oral Infections"), and international application no. PCT/US2004/041083 ("High- level Expression of Fusion Polypeptides in Plant Seeds Utilizing Seed- Storage Proteins as Fusion Carriers"). Other general and specific techniques for producing proteins from plant cells may be obtained, for example, from the following references, each of which is incorporated herein in its entirety by reference: U.S. Patent No. 5,693,507, U.S. Patent No. 5,932,479, U.S Patent No. 6,642,053, and 6,680,426 (each titled "Genetic Engineering of Plant Chloroplasts"); U S. Pat. Pub. No. 2005/0066384 ("Site-Targeted Transformation Using Amplification Vectors"); U.S. Pat. Pub. No. 2005/0221323 ("Amplification Vectors Based on Trans-Splicing"); U.S. Pat. Pub. No. 2006/0026718 ("Method of Controlling Cellular Processes in Plants"); and U.S. Pat. Pub. No. 2006/0075524 (Method of Controlling A Cellular Process in a Multi- Cellular Organism"); Marillonnet et at., Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants, Nature Biotech. (2005) 23(6): 718-723.

Expression vectors include plasmids, episomes, cosmids retroviruses or phages; the expression vector can be used to express a DNA sequence encoding the cell culture media component, and in one aspect comprises an assembly of expression control sequences. The choice of promoter and other regulatory elements can vary according to the intended host cell, and many such elements are available commercially, and can be readily assembled from isolated components such as the Gateway system from Invitrogen, (CA, USA). Expression systems for cell culture media components can be stable or transient expression systems.

Representative commercially available viral expression vectors include, but are not limited to, the adenovirus-based systems, such as the Per.C6 system available from Crucell, Inc., lentiviral-based systems such as pLP1 from Invitrogen, and retroviral vectors such as tobacco mosaic virus based vectors (Lindbo et al., BMC Biotechnol. (2007) 7 52-58)

An episomal expression vector is able to replicate in the host cell, and persists as an extrachromosomal episome within the host cell in the presence of appropriate selective pressure. (See for example, Conese et al., Gene Therapy 11: 1735-1742 (2004)). Representative commercially available episomal expression vectors include, but are not limited to, episomal plasmids that utilize Epstein Barr Nuclear Antigen 1 (EBNA1) and the Epstein Barr Virus (EBV) origin of replication (oriP), specific examples include the vectors pREP4, pCEP4, pREP7 from Invitrogen. The host range of EBV based vectors can be increased to virtually any

eukaryotic cell type through the co-expression of EBNA1 binding protein 2 (EPB2) (Kapoor et al., EMBO. J. 20: 222-230 (2001)), vectors pcDNA3.1 from Invitrogen, and pBK-CMV from Stratagene represent non-limiting examples of an episomal vector that uses T-antigen and the SV40 origin of replication in lieu of EBNA1 and oriP.

An integrating expression vector can randomly integrate into the host cell's DNA, or can include a recombination site to enable the specific recombination between the expression vector and the host cells chromosome. Such integrating expression vectors can utilize the endogenous expression control sequences of the host cell's chromosomes to effect expression of the desired protein. Examples of vectors that integrate in a site specific manner include, for example, components of the flp-in system from Invitrogen (e.g., pcDNA™5/FRT), or the cre-lox system, such as can be found in the pExchange-6 Core Vectors from Stratagene. Examples of vectors that integrate into host cell chromosomes in a random fashion include, for example, pcDNA3.1 (when introduced in the absence of T-antigen) from Invitrogen, pCI or pFN10A (ACT) Flexi® from Promega.

Alternatively, the expression vector can be used to introduce and integrate a strong promoter or enhancer sequences into a locus in the cell so as to modulate the expression of an endogenous gene of interest (Capecchi MR. Nat Rev Genet. (2005); 6 (6):507-12; Schindehutte et al., Stem Cells (2005); 23 (1):10-5). This approach can also be used to insert an inducible promoter, such as the Tet-On promoter (US Patents 5,464,758 and 5,814,618), in to the genomic DNA of the cell so as to provide inducible expression of an endogenous gene of interest. The activating construct can also include targeting sequence(s) to enable homologous or non-homologous recombination of the activating sequence into a desired locus specific for the gene of interest (see for example, Garcia-Otin & Guillou. Front Biosci. (2006) 11:1108-36). Alternatively, an inducible recombinase system, such as the Cre-ER system, can be used to activate a transgene in the presence of 4-hydroxytamoxifen (Indra et al. Nuc. Acid. Res. (1999) 27 (22). 4324-4327; Nuc. Acid. Res. (2000) 28(23). e99; and U.S. Patent No. 7,112,715).

Suitable cells for producing the cell culture media components include prokaryotic cells, yeasts, insect cells, plant expression systems and mammalian expression systems. Within these general guidelines, useful microbial hosts include, but are not limited to, bacteria from the genera Bacillus, Escherichia (such as *E. coli*), Pseudomonas, Streptomyces, Salmonella, Erwinia, *Bacillus subtilis*, *Bacillus brevis*, the various strains of *Escherichia coli* (e.g., HB101, (ATCC NO. 33694) DH5α, DH10 and MC1061 (ATCC NO. 53338)).

Many strains of yeast are known to those skilled in the art and are also available as host cells for the expression of cell culture media components including those from the genera Hansenula, Kluyveromyces, Pichia, Rhino-sporidium, Saccharomyces, and Schizosaccharomyces, and other fungi. Preferred yeast cells include, for example, *Saccharomyces cerivisae* and *Pichia pastoris*.

Additionally, where desired, insect cell systems can be utilized to produce the cell culture media components for use in the supplements and methods of the present invention. Such systems are described, for example, by Kitts et al., Biotechniques, 14:810-817 (1993); Lucklow, Curr. Opin Biotechnol., 4:564-572 (1993); and Lucklow et al (J. Virol., 67:4566-4579 (1993). Preferred insect cells include Sf-9 and Hi5 (Invitrogen, Carlsbad, Calif).

Many suitable plant expression systems can be used for the expression of cell culture media components examples includes for example, any monocot or dicot plant. Suitable monocot plants include without limitation, rice, barley, wheat, rye, corn, millet, triticale, or sorghum, preferably rice. Other suitable plants include Arabidopsis, Alfalfa, tobacco, peanut and soybean.

In general, expression vectors for use in plants include the following operably linked elements that constitute a chimeric gene: a promoter derived from a gene encoding a plant protein, operatively linked to a gene encoding a cell culture media component. The promoter region is chosen to be regulated in a manner allowing for induction under seed-maturation conditions. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, corn, millet, triticale or sorghum.

In one aspect, the chimeric gene includes a promoter which exhibits increased expression during seed maturation. Examples of such promoters include the maturation-specific promoter region associated with one of the following maturation-specific monocot plant storage proteins: rice glutelins, oryzins, and prolamines, barley hordeins, wheat gliadins and glutelins, maize zeins and glutelins, oat glutelins, and sorghum kafirins, millet pennisetins, and rye secalins.

In one aspect, the chimeric gene includes i) a promoter from the gene of a maturation-specific monocot plant storage protein, ii) a first DNA sequence, operably linked to the promoter, encoding a monocot plant seed-specific signal sequence (such as an N-terminal leader sequence or a C-terminal trailer sequence) capable of targeting a polypeptide linked thereto to an endosperm cell, preferably an endosperm-cell organelle, such as a protein storage body, and iii) a second DNA sequence, linked in translation frame with the first DNA sequence, encoding a

cell culture media component. The signal sequence is preferably cleaved from the cell culture media component in the plant cell.

The chimeric gene, in turn, is typically placed in a suitable plant-transformation vector having (i) companion sequences upstream and/or downstream of the chimeric gene which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the desired plant host; (ii) a selectable marker sequence; and (iii) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells.

At least one selective purification tag and/or at least one specific protease cleavage site may be provided for eventual release of the cell culture media component from the monocot seed storage protein carrier. For example, a strategic methionine or tryptophan residue providing a chemical cleavage site may be engineered in frame between the domains for release of the cell culture media component from the endogenous plant protein Other selective protease cleavage sites include, but are not limited to enterokinase (ek), Factor Xa, thrombin, V8 protease, GENENASE™(a variant of subtilisin BPN'), α-lytic protease or tobacco etch virus protease. Alternatively, cleavage of the fusion protein could be performed via chemical cleaving agents such as cyanogen bromide or N-chlorosuccinimide.

A number of suitable mammalian host cells are also known in the art and many are available from the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209. Examples include, but are not limited to, mammalian cells, such as Chinese hamster ovary cells (CHO) (ATCC No. CCL61) CHO DHFR-cells (Urlaub et al., Proc. Natl. Acad Sci. USA, 97:4216-4220 (1980)), human embryonic kidney (HEK) 293 or 293T cells (ATCC No. CRL1573), or 3T3 cells (ATCC No. CCL92). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines are the monkey COS-1 (ATCC No. CRL1650) and COS-7 cell lines (ATCC No. CRL1651), and the CV-1 cell line (ATCC No. CCL70). Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines.

Cell-free transcription and translation systems can also be employed to produce such proteins using the DNA constructs (or RNAs derived from the DNA constructs) of the present invention.

Production of recombinant proteins of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides encoding the cell culture media component and to host cells which are genetically engineered with such expression systems and to the production of such proteins by recombinant techniques. In one embodiment the host cell endogenously expresses a heat shock protein of interest.

In cases where purification of the expressed proteins of the supplement of the invention are necessary, proteins of the present invention can be recovered from either the cellular environment, before lysing the cells, or after cell lysis The proteins can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography. High performance liquid chromatography is also employed for purification.

A search of patents, published patent applications, and related publications will provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying albumin. For example, US Patent Nos. 4,075,197; 4,086,222; 4,093,612; 4,097,473; 4,136,094; 4,228,154; 5,250,662; 5,656,729; 5,677,424; 5,710,253; 5,728,553; 5,994,507; 6,001,974; 6,638,740; 6,617,133 and 7,423,124 disclose various processes for purifying albumin. In one aspect, the albumin for use in the present invention is purified using any of these art recognized processes listed above, and then mixed in aqueous solution with a heat shock protein Methods for the purification of heat shock proteins, including anion exchange chromatography and ATP agarose affinity chromatography are well known in the art. (Welch & Feramisco, J. Biol. Chem. 257 (24)14949-14959; (1982); Welch & Feramisco, Mol. Cell. Biol. 5 (6) 1229-1237 (1985) Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification

In one preferred aspect recombinant albumin is purified using procedures that enable the direct co-purification of both recombinant albumin and a heat shock protein, or hsp protein complex, for example as described below and in the Examples section In one aspect the recombinant albumin is produced in rice, and the heat shock protein is an endogenous rice heat shock protein.

In one aspect, of this method, expression vectors are used to increase the expression of the recombinant albumin in the host cell, while the expression of the host cells endogenous heat shock proteins is accomplished by activating the expression of the host cells hsp genes. In another aspect expression vectors are used to increase the expression of the heat shock protein. In another aspect expression vectors are used to increase the expression of the heat shock protein and albumin. In another aspect the nucleic acid sequence encoding a heat shock protein and albumin are located in the same expression vector

Due to the similar electronegativity of albumin and hsp70, anion exchange chromatography is the preferred method to prepare albumin enriched in Hsps. For example, both albumin and Hsp70 bind to anion exchange columns with resins consisting of either quaternary amine or diethylaminoethyl mounted on a bead that is suitable for the ion exchange of polypeptides (large molecular exclusion limit and of suitable size) at high pH (7.5 and above). Examples of such resins are General Electric (GE) Q Sepharose and GE DEAE Sepharose. Due to their similar electronegativity, utilizing low pH conditions (below pH 6.5) allows for the co-purification of the two molecules on cation exchangers as well. Examples of such cation exchangers are GE Carboxymethyl Sepharose and Sulfonic acid Sepharose based resins. Because the albumin and Hsp70 have similar isoelectric points, mixed mode resins may also be employed for the co-purification of albumin and Hsp70. Since both Hsp70 and Albumin are well known to bind to fatty acids and other hydrophobic molecules, it is also possible to co-purify albumin and Hsp70 on a hydrophobic based resin such as octyl sepharose (GE). Due the similar size of Hsp70 proteins and Albumin (65-75 kDa), co-purification of the two proteins and enrichment of Hsp70 by tangential flow ultrafiltration utilizing both higher and lower molecular exclusions than 65-75 kDa may also be employed to co-purify and thus enrich Albumin with hsps.

Also due to their similar molecular weights, any method that separates polypeptides based on size should effectively co-purify albumin and hsp70 such as molecular sieves and gel filtration or size exclusion chromatography. In addition, due to the similar nature of Hsp70 and Albumin in terms of hydrophobicity and electronegativity or surface charge may be co-purified by precipitation under a number of conditions. Some of those conditions are precipitation by ammonium sulfate, precipitation by denaturants such as urea, or precipitation based on isoelectric point and solubility

The methods are also applicable to enrich albumin with hsps from other sources. For example albumin derived from native and transgenic animal feedstock serum, as well as albumin

produced from recombinant organisms and tissue culture systems based on prokaryotic and eukaryotic cells, including, vertebrate cells such as mammalian cells, and non vertebrate cells, such as insects, as well as plant, and fungi such as yeast, and the like

A search of patents, published patent applications, and related publications will also provide those skilled in the art reading this disclosure with significant possible methods for preparing and purifying transferrin related proteins. For example, US Patent Nos 7,368,141; 7,354,902; 5,708,149. 5,596,082; 5,169,936; 4,791,193; and 4,667,018 disclose various processes for purifying transferrin related proteins

IV Exemplary Cells

Without wishing to be bound by theory, it is contemplated that any cell which is susceptible to apoptosis may be used in the methods of the invention, including primary cells, immortalized cells, differentiated cells, undifferentiated cells or cells, such as stem cells, with varying degrees of specialization. In a particular embodiment, cells used in the methods of the invention are transfected with a nucleic acid molecule comprising a nucleotide sequence encoding a protein of interest, e.g., a therapeutic protein or an antibody.

In a particular embodiment, the cells used in the methods of the invention are eukaryotic cells, e.g., mammalian cells. Examples of mammalian cells include, but are not limited to, for example, human B-cells, and T cells, and derivatives thereof, such as hybridomas, and cell expressing markers of B or T cells, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J Gen Virol., 36:59 (1977)), baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); CHO-K1 cell (ATCC CCL-61), human PER.C6 cells (Crucell, NV), mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci., 383:44-68 (1982)). MRC 5 cells; FS4 cells; NSO mouse myeloma cells (ECACC; SIGMA), and a human hepatoma line (Hep G2) Additional examples of useful cell lines include, but are not limited to, HT1080 cells (ATCC CCL 121), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB

carcinoma cells (ATCC CCL 17), 2780AD ovarian carcinoma cells (see Van der Blick, A M. et al , Cancer Res. 48:5927-5932 (1988), Raji cells (ATCC CCL 86), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells (ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607), WI-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. These and other cells and cell lines are available commercially, for example from the American Type Culture Collection (Virginia, USA) Many other cell lines are known in the art and will be familiar to the ordinarily skilled artisan; such cell lines therefore can be used equally well in the methods of the present invention. In a particular embodiment, cells used in the methods of the invention are CHO cells or NSO cells. Hybridomas and antibody-producing cells may also be used in the methods of the invention

In another embodiment, cells used in any of the methods of the invention are stem cells. Stem cells are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny cells, including self renewing progenitors, non-renewing progenitors, and terminally differentiated cells. Stem cells are also characterized by their ability to differentiate in vitro into functional cells of various cell lineages from multiple germ layers (endoderm, mesoderm and ectoderm), as well as to give rise to tissues of multiple germ layers following transplantation and to contribute substantially to most, if not all, tissues following injection into blastocysts

Types of human embryonic stem cells that may be used in any of the methods of the invention include established lines of human embryonic cells derived from tissue formed after gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Non-limiting examples are established lines of human embryonic stem cells or human embryonic germ cells, such as, for example the human embryonic stem cell lines H1, H7, and H9 (WiCell). Also contemplated is use of the compositions of this disclosure during the initial establishment or stabilization of such cells, in which case the source cells would be primary pluripotent cells taken directly from the source tissues. Also suitable are cells taken from a pluripotent stem cell population already cultured in the absence of feeder cells. Also suitable are mutant human embryonic stem cell lines, such as, for example, BG01v (BresaGen, Athens, Ga). In one embodiment, Human embryonic stem cells are prepared as described by Thomson et al. (U.S. Pat. No. 5,843,780: Science 282:1145, 1998; Curr Top Dev Biol. 38:133 ff., 1998; Proc. Natl Acad. Sci. U.S.A. 92:7844, 1995)

Additionally, hybridoma cells can also be used in the methods of the invention. The term "hybridoma" refers to a hybrid cell line produced by the fusion of an immortal cell line of immunologic origin and an antibody producing cell. The term encompasses progeny of heterohybrid myeloma fusions, which are the result of a fusion with human cells and a murine myeloma cell line subsequently fused with a plasma cell, commonly known as a trioma cell line. Furthermore, the term is meant to include any immortalized hybrid cell line which produces antibodies such as, for example, quadromas See. e.g., Milstein et al., Nature. 537:3053 (1983) The hybrid cell lines can be of any species, including human, rabbit and mouse.

In some embodiments, a cell line used in the methods of the invention is an antibody-producing cell line. Antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan See, e g., Current Protocols in Immunology, Coligan et al., Eds , Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements. In general, any cell suitable for recombinant protein expression in cell culture can be used in the methods of the invention.

In some embodiments, the cells used in the methods of the present invention may include a heterologous nucleic acid molecule which encodes a desired recombinant protein, e.g., a therapeutic protein or antibody which is desired to be produced using the methods of the invention. In a particular embodiment, the methods of the present invention are useful for producing high titers of a desired recombinant protein, e.g., a therapeutic protein or antibody, in the presence of reduced levels of one or more contaminants.

V. Cell Culture Media

Any suitable culture medium or feed medium suitable for cell growth and protein production may be used in the methods of the invention. Suitable culture or feed mediums are chosen for their compatibility with the host cells and process of interest. Suitable culture or feed mediums are well known in the art and include, but are not limited to, commercial media such as Ham's F10 (SIGMA), Minimal Essential Medium (SIGMA), RPMI-1640 (SIGMA), and Dulbecco's Modified Eagle's Medium SIGMA) are suitable for culturing the animal cells. In addition, any of the media described in Ham and Wallace, (1979) Meth. Enz . 58:44; Barnes and Sato,(1980) Anal Biochem., 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469 or 4,560,655; International Publication Nos. WO 90/03430; and WO 87/00195 may be used.

Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The necessary growth factors for a particular cell are readily determined empirically without undue experimentation, as described for example in Mammalian Cell Culture (Mather, J. P ed., Plenum Press, N.Y (1984), and Barnes and Sato, Cell, 22:649 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the protein of interest in recombinant vertebrate cell culture are described in Gething et al , Nature, 293.620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117.058. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, M. Butler, ed. (IRL Press, 1991).

VI. Exemplary Cell Culture Expression Products

In one aspect of any of the claimed methods, the supplements of the invention are used to improve the viability and growth of a cell which is used to express and produce a protein of interest. The cell may express the protein of interest endogenously or may be an engineered cell line that has been modified genetically to express the protein of interest at levels above background for that cell.

Cells may be genetically modified to express a protein by transformation with a nucleic acid encoding the protein of interest, or by transformation of an activating sequence that promotes the expression of an endogenous gene. In one aspect the protein of interest may be expressed from an expression vector, in which a coding sequence for the protein of interest is operably linked to an expression control sequences, to enable either constitutive or inducible expression, as is known in the art

The protein of interest may be any protein, or fragment thereof, which is of commercial, therapeutic or diagnostic value including without limitation cytokines, chemokines, hormones, antibodies, anti-oxidant molecules, engineered immunoglobulin-like molecules, a single chain antibodies, a humanized antibodies, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, transdominant negative mutants of a target protein, toxins, conditional toxins, antigens, a tumor suppresser proteins, growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group). The protein of interest may also comprise pro-drug activating enzymes.

In some embodiments, the protein of interest comprises a glycoprotein, or any other protein which has one or more post-translational modifications. For example, any protein which is suitable for production in a eukaryotic host may be expressed using the methods and compositions described here.

The methods of the invention can be used to produce any desired recombinant protein or fragment thereof. In some embodiments, a recombinant protein produced using the methods described herein is a therapeutic protein. In other embodiments, the recombinant protein is an antibody or functional fragment thereof. Antibodies which may be produced using the methods of the invention include, for example, polyclonal, monoclonal, monospecific, polyspecific, fully human, humanized, single-chain, chimeric, hybrid, CDR grafted,. It may comprise a full length IgG1 antibody or an antigen-binding fragments thereof, such as, for example, Fab, $F(ab')_2$, Fv, and scfv.

Antibodies within the scope of the present invention include, but are not limited to, anti-HER2 antibodies including Trastuzumab (HERCEPTIN™) (Carter et al. Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN™), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108, B1, or Tositumomab (BEXXAR™.); anti-IL-8 (St John et al., Chest, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN™ (Kim et al., Growth Factors, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., Transplant Intl. 4:3-7 (1991), and Hourmant et al., Transplantation

58:377-380 (1994)); anti-IgE (Presta et al., J. Immunol. 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No PCT/US98/13410 filed Jun 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-alpha, antibodies including cA2 (REMICADE™), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. J. Immunol. 156(4).1646-1653 (1996), and Dhainaut et al Crit. Care Med. 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human alpha 4 beta 7 integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S Pat No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT™) and (ZENAPAX™) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al Arthritis Rheum 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. Nature 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fc gamma RI as in Graziano et al. J. Immunol. 155(10):4996-5002 (1995), anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. Cancer Res. 55(23Suppl): 5935s-5945s (1995), antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. Cancer Res. 55(23). 5852s-5856s (1995); and Richman et al. Cancer Res. 55(23 Supp). 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. Eur J. Immunol. 26(1):1-9 (1996)), anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. J. Immunol. 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. Cancer Res 55(23 Suppl).5908s-5910s (1995) and CMA-676 or CDP771, anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. Cancer Res 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX™.); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO™ anti-RSV antibodies such as MEDI-493 (SYNAGIS™.); anti-CMV antibodies such as PROTOVIR™.; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR™; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-.alpha.v.beta.3 antibody VITAXIN™; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33), anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a,

and CD40.

The recombinant protein may be a cellular protein such as a receptor (e.g., membrane bound or cytosolic) or a structural protein (e.g. a cytoskeleton protein). The recombinant protein may be cellular factor secreted by the cell or used internally in one or more signal transduction pathways. Non limiting examples include, but are not limited to, CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1, IL-2, IL-3, IL-7, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF, EGF receptor, VEGF receptor, hepatocyte growth factor, osteoprotegerin ligand, interferon gamma, B lymphocyte stimulator C5 complement TAG-72, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2, and CTLA4 (which is a cytotoxic T lymphocyte-associated antigen).

The recombinant protein may also be derived from an infectious agent such as a virus, a bacteria, or fungus. The protein may be derived from a bacterial membrane or cell wall, or may be derived from the bacterial cytosol. The protein may be a bacterial or yeast enzyme, transcription factor, or structural protein. The bacterial or yeast protein may be membrane bound, cytsolic, or secreted. Examples of infectious agents include, but are not limited to, Streptococcus mutans, and Staphlycoccus aureus, and Candida albicans.

The methods of the invention can also be used to produce recombinant fusion proteins comprising all or part of any of the above-mentioned proteins. For example, recombinant fusion proteins comprising one of the above-mentioned proteins plus a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an antibody, or a substantially similar protein, can be produced using the methods of the invention. See e.g. International Application No. WO 94/10308; Lovejoy et al. (1993), Science 259:1288-1293; Harbury et al. (1993), Science 262: 1401-05; Harbury et al. (1994), Nature 371:80-83; Hang.kansson et al. (1999), Structure 7:255-64.

Also encompassed by this invention are pharmaceutical compositions including one or more recombinant proteins produced by the methods described herein. In some embodiments, pharmaceutical compositions further include a pharmaceutically acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or

liquid filler, diluents or encapsulating substances which are suitable for administration into a subject.

VII Stem Cells

In one embodiment, human embryonic stem cells are cultured in a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation, comprising a supplement of the invention The growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a medium conditioned by culturing previously with another cell type and further comprising a supplement of the present invention. Alternatively, the growth of human embryonic stem cells in feeder-free culture without differentiation is supported using a chemically defined medium comprising a supplement of the present invention. Examples of feeder-free, serum free culture systems in which embryonic stem cells are maintained in unconditioned serum replacement (SR) medium supplemented with different growth factors capable of triggering embryonic stem cell self-renewal include those disclosed in US patent applications, US20050148070, US20050244962, US20050233446, U.S. Pat. No.6,800,480, and PCT publications WO2005065354 and WO2005086845.

In an alternate embodiment, human embryonic stem cells are initially cultured with a layer of feeder cells that support the human embryonic stem cells and further comprising a supplement of the present invention The human embryonic are then transferred to a culture system that is essentially free of feeder cells, but nonetheless supports proliferation of human embryonic stem cells without undergoing substantial differentiation and which further comprises a supplement of the present invention In any of these approaches, the use of the supplements of the invention results in significantly enhanced rates of cell growth and improved cell viability.

Examples of conditioned media suitable for use with the supplements of the present invention are disclosed in US20020072117, U.S. Pat. No. 6,642,048, WO2005014799, and Xu et al (Stem Cells 22: 972-980, 2004) An example of a chemically defined medium suitable for use with the supplements of the present invention may be found in US20070010011.

Examples of feeder cells include feeder cells selected from the group consisting of a fibroblast cell, a MRC-5 cell, an embryonic kidney cell, a mesenchymal cell, an osteosarcoma cell, a keratinocyte, a chondrocyte, a Fallopian ductal epithelial cell, a liver cell, a cardiac cell, a bone marrow stromal cell, a granulosa cell, a skeletal muscle cell, a muscle cell and an aortic

endothelial cell. In a preferred embodiment, the MRC-5 cell, has ATCC Catalog Number 55-X; the transformed and has ATCC Accession Number CRL-2309; the human osteosarcoma cell has ATCC Accession Number HTB-96; and the mesenchymal cell is a human fetal palatal mesenchymal cell with ATCC Accession Number CRL-1486. In other preferred embodiments the human fibroblast cell is a skin keloid fibroblast, KEL FIB and has ATCC Accession Number CRL-1762, or is a fetal skin fibroblast cell; and the bone marrow stromal cell, HS-5, has ATCC Accession Number CRL-11882.

Suitable culture media may be made from the following components, such as, for example, Dulbecco's modified Eagle's medium (DMEM), Gibco # 11965-092; Knockout Dulbecco's modified Eagle's medium (KO DMEM), Gibco # 10829-018; Ham's F12/50% DMEM basal medium; 200 mM L-glutamine, Gibco #15039-027; non-essential amino acid solution, Gibco 11140-050; β-mercaptoethanol, Sigma # M7522, human recombinant basic fibroblast growth factor (bFGF), Gibco # 13256-029.

In one embodiment, the human embryonic stem cells are plated onto a suitable culture substrate that is treated prior to treatment according to the methods of the present invention, with a composition comprising a supplement of the present invention. In one embodiment, the treatment is an extracellular matrix component, such as, for example, those derived from basement membrane or that may form part of adhesion molecule receptor-ligand couplings. In one embodiment, the suitable culture substrate is MATRIGEL (Becton Dickenson). MATRIGEL is a soluble preparation from Engelbreth-Holm-Swarm tumor cells that gels at room temperature to form a reconstituted basement membrane.

Other extracellular matrix components and component mixtures are suitable as an alternative and can be used with the supplements of the present invention. This may include laminin, fibronectin, proteoglycan, entactin, heparan sulfate, and the like, alone or in various combinations with a supplement of the present invention.

In another embodiment, the invention encompasses an embryonic stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention. In one embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a feeder-free, serum free culture system comprising a supplement of the invention

In another embodiment the invention encompasses an embryonic stem cell culture, comprising a human embryonic stem cell and a human feeder cell culture comprising a supplement of the

invention. In another embodiment the invention encompasses a human pluripotent stem cell culture, comprising a human pluripotent stem cell and a human feeder cell culture comprising a supplement of the invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing pluripotency markers, comprising the steps of:

Culturing human embryonic stem cells,

Differentiating the human embryonic stem cells into cells expressing pluripotency markers, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In another embodiment, the present invention provides a method for deriving a population of cells comprising cells expressing markers, characteristic of ectodermal, endodermal or mesodermal cells, comprising the steps of:

Culturing pluripotency stem cells;

Differentiating the pluripotency stem cells into cells expressing markers characteristic of ectodermal, endodermal or mesodermal cells, wherein the differentiation is conducted in the presence of a supplement of the present invention.

In any of these methods, the stem cells can be differentiated into cells expressing markers characteristic of an endodermal, ectodermal or mesodermal lineage by any method in the art. For example, cells expressing pluripotency markers may be differentiated into cells expressing markers characteristic of the definitive endoderm lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 23, 1534-1541 (2005), by Shinozaki et al, Development 131, 1651-1662 (2004), McLean et al., Stem Cells 25, 29-38 (2007), D'Amour et al., Nature Biotechnology 24, 1392-1401 (2006).

Cells expressing markers characteristic of the endoderm lineage may be further differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage by any method in the art. For example, cells expressing markers characteristic of the pancreatic endoderm lineage may be differentiated into cells expressing markers characteristic of the pancreatic endocrine lineage according to the methods disclosed in D'Amour et al, Nature Biotechnology 24, 1392-1401 (2006), wherein the differentiation is conducted in the presence of a supplement of the present invention.

In one aspect of any of these methods of differentiation, the human embryonic stem cells are

cultured and differentiated on a tissue culture substrate coated with an extracellular matrix. The extracellular matrix may be a solubilized basement membrane preparation extracted from mouse sarcoma cells (which is sold by BD Biosciences under the trade name MATRIGEL). Alternatively, the extracellular matrix may be growth factor-reduced MATRIGEL. Alternatively, the extracellular matrix may be fibronectin. In an alternate embodiment, the human embryonic stem cells are cultured and differentiated on tissue culture substrate coated with human serum. In one aspect, the tissue culture substrate is coated with extracellular matrix and a supplement of the present invention.

The extracellular matrix may be diluted prior to coating the tissue culture substrate. Examples of suitable methods for diluting the extracellular matrix and for coating the tissue culture substrate may be found in Kleinman, H. K., et al , Biochemistry 25:312 (1986), and Hadley, M A., et al., J. Cell. Biol 101:1511 (1985).

In one aspect of the methods of stem cell differentiation, the culture medium should contain sufficiently low concentrations of certain factors to allow the differentiation of human embryonic stem cells to cells of endoderm, ectoderm or mesoderm lineage. such as, for example insulin and IGF (as disclosed in WO2006020919). This may be achieved by lowering the serum concentration, or alternatively, by using chemically defined media that lacks insulin and IGF. Examples of chemically defined media are disclosed in Wiles et al (Exp Cell Res. 1999 Feb. 25, 247(1): 241-8.). In a preferred embodiment, of any of these methods. the culture media comprises a supplement of the present invention.

The culture medium may also contain at least one other additional factor that may enhance the formation of cells expressing markers characteristic of endoderm, mesoderm or ectoderm lineage from human embryonic stem cells. The at least one additional factor may be, for example, nicotinamide, members of TGF-β family, including TGF-β1, 2, and 3, serum albumin, members of the fibroblast growth factor family, platelet-derived growth factor-AA, and -BB, platelet rich plasma, insulin growth factor (IGF-I, II), growth differentiation factor (GDF-5, -6, -8, -10, 11), glucagon like peptide-I and II (GLP-I and II), GLP-1 and GLP-2 mimetobody, Exendin-4, retinoic acid, parathyroid hormone, insulin, progesterone, aprotinin, hydrocortisone, ethanolamine, beta mercaptoethanol, epidermal growth factor (EGF), gastrin I and II, copper chelators such as, for example, triethylene pentamine, forskolin, Na-Butyrate, activin, betacellulin, ITS, noggin, neurite growth factor, nodal, valproic acid, trichostatin A, sodium butyrate, hepatocyte growth factor (HGF), sphingosine 1, VEGF, MG132 (EMD, CA), N2 and B27 supplements (Gibco, CA), steroid alkaloid such as, for example, cyclopamine (EMD, CA),

keratinocyte growth factor (KGF), Dickkopf protein family, bovine pituitary extract, islet neogenesis-associated protein (INGAP), Indian hedgehog, sonic hedgehog, proteasome inhibitors, notch pathway inhibitors, sonic hedgehog inhibitors, or combinations thereof. In a preferred embodiment, of any of these methods, the culture media containing at least one additional factor listed above, further comprises a supplement of the present invention.

The at least one other additional factor may be supplied by conditioned media obtained from pancreatic cells lines such as, for example, PANC-1 (ATCC No: CRL-1469), CAPAN-1 (ATCC No: HTB-79), BxPC-3 (ATCC No. CRL-1687), HPAF-II (ATCC No: CRL-1997), hepatic cell lines such as, for example, HepG2 (ATCC No: HTB-8065), and intestinal cell lines such as, for example, FHs 74 (ATCC No: CCL-241). In a preferred embodiment, of any of these methods, the conditioned media further comprises a supplement of the present invention.

In another embodiment, the invention encompasses a method of using the cell or tissue of any of the aforementioned stem cells for the experimental, therapeutic and prophylactic treatment of a disease or condition in a human or animal. Preferably, the disease is selected from the group consisting of Parkinson's, Alzheimer's, Multiple Sclerosis, spinal cord injuries, stroke, macular degeneration, burns, liver failure, heart disease, diabetes, Duchenne's muscular dystrophy, osteogenesis imperfecta, osteoarthritis, rheumatoid arthritis, anemia, leukemia, breast cancer, solid tumors, and AIDS. In a preferred embodiment, the disease is Parkinson's or Alzheimer's. In a more preferred embodiment, the disease is Parkinson's.

VIII. Large Scale production of recombinant proteins

In one embodiment, the supplements of the present invention can be used to produce a protein of interest by growing host cells in the presence of the supplement. In one embodiment, the cell culture is performed in a stirred tank bioreactor system and a fed batch culture procedure is employed. In another embodiment a wave disposable bioreactor is employed. In the bioreactor system, the size of the bioreactors are sufficiently large to produce the desired amount of protein of interest, such as 1,000 Liter or 12,000 Liter sizes, but are not limited to such sizes as much smaller (i.e., 2 Liter, 400 Liter) or larger (i.e., 25,000 Liter, 50,000 Liter) bioreactor vessels may be appropriate. In the preferred fed batch culture, the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells

and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process but at the termination of the culture process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cultured cells may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single step or multiple step culture procedure. In a single step culture, the host cells are inoculated into a culture environment and the method steps of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture, cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium comprising a supplement of the present invention suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase, cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 38° C. and preferably about 37° C. and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above, the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell culture environment during the production phase of the cell culture is controlled. According to the steps of the presently disclosed methods, the

addition of the supplements of the invention can be coordinated such that the desired content and quality of the protein of interest is achieved and maintained in the resulting cell culture fluid. In a preferred aspect, the production phase of the cell culture is preceded by a transition phase of the cell culture in which the addition of the supplements of the invention initiates the production phase of the cell culture.

In any of the above-described methods, it is contemplated that it may be desirable to include a desired amount of agent like butyrate or Trichostatin A in the cell culture medium in combination with a supplement of the invention. Various forms of butyrate and its salts are known in the art, such as butyric acid and sodium butyrate, and are publicly available from sources such as Sigma Chemical Co. Butyrate has been reported in the literature to enhance the productivity and protein expression of cell cultures [Arts et al , Biochem J., 310:171-176 (1995), Gorman et al , Nucleic Acids Res., 11.7631-7648 (1983); Krugh, Mol. Cell Biochem. 42:65-82 (1982); Lamotte et al., Cytotechnology, 29:55-64 (1999); Chotigeat et al., Cytotechnology, 15:217-221 (1994)]. Trichostatin A (TSA) is an inhibitor of histone deacetylase and may act similarly to butyrate in enhancing the productivity and protein expression in cell cultures [Medina et al., Cancer Research, 57:3697-3707 (1997)]. Although butyrate has some positive effects on protein expression, it is also appreciated in the art that at certain concentrations, butyrate can induce apoptosis in the cultured cells and thereby decrease viability of the culture as well as viable cell density [Hague et al., Int. J. Cancer, 55:498-505 (1993); Calabresse et al , Biochim. Biophys. Res. Comm., 195:31-38 (1993), Fillipovich et al., Biochim Biophys. Res. Comm., 198:257-265 (1994), Medina et al., Cancer Research, 57:3697-3707 (1997)]. In the methods of the present invention, a desired amount of butyrate or TSA may be added to the cell culture at the onset of the production phase and more preferably, may be added to the cell culture after a temperature shift has been implemented Butyrate or TSA can be added in a desired amount determined empirically by those skilled in the art, but preferably, butyrate is added to the cell culture at a concentration of about 1 to about 25 mM, and more preferably, at a concentration of about 1 to about 6 mM.

Expression of the protein of interest may be measured in a sample directly, for example, by ELISA, conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a

wide variety of labels, such as radionucleotides, fluorophors or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal and may be prepared in any mammal. Many are commercially available.

The supplements claimed herein can also be used to increase transfection efficiency and viability of cells during transfection. Conditions and reagents used in various transfection techniques, such as Lipofectamine are relatively toxic to the cells, while electroporation can severely stress a cell. The use of higher concentrations of transfection reagents, and more extensive electroporation conditions is preferred to achieve higher transfection efficiencies. Thus the addition of the supplements of the invention prior, with, and after transfection can result in higher transfection efficiencies, and higher yields of recombinant proteins.

The supplements of the invention can be used to express proteins of interest which induce apoptosis, such as Apo-2 ligand / TRAIL or Fas ligand. The presence of the supplements of the invention may block such apoptotic activity and allow for improved expression of the protein of interest.

In addition, the methods can be used to increase the viability of cells undergoing freezing / storage/thawing procedures. During these procedures generally cells can lose viability. The presence of apoptosis inhibitors added to the cell culture media can provide for increased cell viability and aid in reducing or eliminating the variability in cell viabilities between aliquots or

vials of cells.

IX. Kits

Also encompassed by the present invention are kits for promoting the viability of cells. In one embodiment, a kit according to the present invention comprises: (a) one or more reagents or devices for transfection and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding using the transfection device, transfection agent and supplement.

In another embodiment a kit according to the present invention comprises: (a) one or more reagents or devices for freezing or thawing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for freezing or thawing cell lines and the use of the reagents.

In another embodiment a kit according to the present invention comprises: (a) one or more tissue culture products for culturing cells and (b) a supplement of the present invention. In some embodiments, kits featured herein include instructions and/or promotional materials including details regarding protocols for dilution cloning techniques and the use of the reagents in such approaches.

All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and indicated individually to be incorporated by reference in its entirety. The following examples illustrate but are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Expression of recombinant human lactoferrin.

A. An Expression Vector For Human Lactoferrin Expression In Transgenic Rice

Complete nucleotide sequence of human mammary gland lactoferrin was codon optimized and synthesized by Operon Technologies (CA, USA). Human milk lactoferrin gene (Genbank accession number: HSU07642) was re-synthesized with codons most frequently used in translation of rice seed proteins in order to obtain optimal level of expression (**FIG. 1A-FIG. 1D**).

Although numbers of codons changed accounted for 22 46% of the entire sequence, amino acid composition remains identical to native human lactoferrin. The plasmid containing the codon-optimized gene was called Lac-ger Lac-ger was digested with SmaI/XhoI and the fragment containing the lactoferrin gene was cloned into pAPI141 that was partially digested with NaeI and completely digested with XhoI. For expression of hLF in rice seeds, the codon-optimized gene was operably linked to the rice endosperm-specific glutelin (Gt1) promoter and NOS terminator. The resulting plasmid was designated pAPI164 (**FIG. 2**).

B. Production system

Rice variety Taipei 309 (Oryza sativa, Japonica) was selected as the production system for recombinant human lactoferrin and transgenic rice events were generated by the particle bombardment of embryogenic rice calli with the plasmid pAPI164 and a companion marker plasmid containing the hygromycin phosphotransferase gene as a selectable marker. Fully developed, fertile rice plants were obtained by this procedure

C. High Level Protein Expression of recombinant human lactoferrin in rice grain

Expression of recombinant human lactoferrin was under the control of the seed maturation-specific promoter Gt1. The high level expression of recombinant human lactoferrin is evident in **FIG. 3** as independent transgenic rice events were screened. Total soluble proteins from mature rice seed extracts were run on Laemli gels and stained with Coomassie blue to visualize the proteins. An ~80kD recombinant lactoferrin protein was obtained in all transgenic lines as indicated by the stained gel. Expression levels of recombinant human lactoferrin corresponded to 0.5% of seed weight. The stable expression of recombinant human lactoferrin was monitored for 10 generations. The expression level is maintained at approximately 0.5% of seed weight of brown rice (**FIG. 4**).

Example 2: Expression & purification of recombinant human transferrin.

Purification of recombinant human from rice grain.

The rice grain is dehusked and milled to an average of 100 mesh flour using standard food industry procedures. Hulls and the solid material remaining after the extraction described below are destroyed. Recombinant human transferrin is extracted from the flour with extraction buffer (20 mM Tris-Base, pH 7.5), with stirring, for 40 minutes at room temperature. Filter aid (Cellpure 300) is added at 20g/L to the extraction mixture before filtration through a filter press with 10 kDa regenerated cellulose.

The filter press filtrate was diafiltered and concentrated with a 30 KDa molecular weight cutoff cellulose membrane into10 mM Tris-Base pH. 8.5 to prepare for DEAE chromatography. The DEAE column was washed with (20mM Tris-Base, 40mM Sodium Chloride, pH 7 5) prior to loading the concentrate onto the column. After loading, the column was washed with 3 volumes of 10 mM Tris-Base, 0.5% (v/v) Triton X-114, pH 8.5 buffer followed by 7 volumes of 10 mM Tris-Base, pH 8.5 buffer. Transferrin was then eluded with 20mM Tris-Base, 40mM Sodium Chloride, pH 7.5.

Transfernn that was recovered from the DEAE column was diafiltered and concentrated with a 10 KDa molecular weight cut off cellulose membrane into 10 mM sodium phosphate, 25mM sodium chloride, pH 7 0 buffer. After diafiltration, recombinant transferrin was filtered though a 0.2 u filter prior to drying.

Example 3: Expression of recombinant human albumin in rice grain.

Protein sequences of human albumin (HSA) from various data bases were compared. The consensus sequence represented by accession number P02768 (Swiss-Prot) was used as a base for gene codon-optimization for suitable expression of human albumin in rice grain (**FIG. 5A-FIG. 5D**). Gene synthesis was carried out by Blue Heron (Seattle, WA) and the synthetic fragment was inserted into pUC based vector to create pUC-HSA. After confirmation of correct DNA and protein sequences, pUC-HSA was digested with MlyI and XhoI. The fragment containing codon-optimized HSA gene was inserted into pAPI405, which had been precut with NaeI and XhoI. Plasmid API405 was a derivative of pAPI141 which included Gt1 promoter, Gt1 signal sequence and a nos terminator.

Insertion of MlyI/XhoI fragment into pAPI405 resulted in pAPI504 (**FIG. 6**). Plasmid API504 was then cleaved with HindIII and EcoRI. The HindIII/EcoRI fragment containing the entire expression cassette, Gt1 promoter, Gt1 signal sequence, codon-optimized HSA gene and nos terminator, was cloned into pJH2600 predigested with the same enzyme resulting in pAPI508 (**FIG. 7**). Plasmid JH2600 was a shuttle vector between E coli and Agrobacterium. After DNA sequence verification, pAPI508 was moved into Agrobacterium AGL-1 for rice transformation. Plasmid API504 was also used via bombardment-based transformation following the procedure described previously. Upon transformation, transgenic plants were generated and were sent to greenhouse where transgenic R0 plants grew to maturity and set R1 seeds.

To monitor the expression of HSA in rice seeds. 10 R1 seeds from each R0 plant were

extracted using 10 mL of extraction buffer (50 mM Tris-HCl pH 8.0. 150 mM NaCl). The supernatant was collected and the expression level from rice extracts was monitored by an ELISA (Bethyl Laboratories, Montgomery, TX) The results showed that the HSA expression level in rice transgenic seeds ranges from 0.01 to 0.85% of brown flour weight (0.1 to 8.5 grams/kg flour). The results of the eight events with highest expression levels are shown in the following table (**Table E1**).

Table E1 Recombinant HSA expression level in top eight events

Line Number	Express Level (g/kg flour)
508-30	5.5
508-17	8.0
508-71	8.7
508-73	4.0
508-77	8.5
508-83	8.5
508-101	3 0
508-113	4.0

Example 4: Purification of recombinant human lactoferrin as cell culture media component.

To prepare cell culture media supplemented with recombinant human lactoferrin, recombinant human lactoferrin was purified from rice flour. Transgenic rice line (164-12) expressing high levels of recombinant human LF was selected. This line. now named as LF164, was planted two generations per year, alternating field planting in summer and greenhouse planting in winter. For protein purification, paddy rice expressing rhLF was de-hulled (Rice Mill, PS-160, Rimac, FL), and then ground to flour (average particle size of 100 mesh) using a hammer mill (8WA. Schutte-Buffalo, NY).

Protein extraction from transgenic flour was performed by mixing two kg of rice flour and 20 L of extraction buffer (0.02 M sodium phosphate pH 6.5 and 0.3 M sodium chloride) in a 50 L tank for 1 h. At the end of the mixing period, the suspension was allowed to settle overnight or centrifuged at 3750 rpm. In both cases, the supernatant was filtered through a plate and frame filter press (Ertel Alsop, 8S. NY) using M-05 and M-70 cellulose/perlite-based filters (Ertel Alsop. NY), respectively.

The filtrate containing rhLF and other rice flour soluble proteins was loaded onto an ion exchange column for further purification. An INDEX 200/500 process column (Amersham Pharmacia Biotech, NJ) packed with SP-Sepharose fast flow (Amersham Pharmacia Biotech, NJ) was used. The column was run at a linear flow rate of 150-200 cm/h. Packing, cleaning and testing of the resin bed was executed per manufacturer's instruction (HETP Test). The filtrate was loaded on to the resin at a linear velocity of 175 cm/h and washed with 0.02 M sodium phosphate buffer (pH 6.5) containing 0.3 M NaCl until the A_{280} returned to baseline. Recombinant hLF was eluted using 20 mM sodium phosphate buffer (pH 6.5) containing 0.8 M NaCl. The washing and elution were performed at 200 cm/h and 150 cm/h, respectively.

A Centramate module (Pall Biopharmaceutical, MA) with 1 ft^2 50 kDa polyethersulfone (Pall Biopharmaceutical, MA) membrane was used for concentration and desalting (ultrafiltration) of eluted hLF. The filtration was performed at a cross flow rate of approximately 1.5 L/min and an average transmembrane pressure of 10 psig. The eluted rhLF was concentrated and desalted to a final volume of 0.25 L and then lyophilized dry. Usually, about 3 grams of purified recombinant human lactoferrin was recovered from one kilogram of transgenic rice flour.

The recombinant human lactoferrin purified from rice flour is approximately 50% saturated with iron (partial-lactoferrin). The 50% saturated recombinant human lactoferrin was then made >90% iron saturated by iron up taking treatment, resulting in holo-lactoferrin and was made <10% iron saturated by acid treatment to remove bound iron resulting in apo-lactoferrin. The purified lactoferrins (holo-, partial- and apo-) were used as cell culture media components.

Example 5: Purification and characterization of recombinant serum human albumin (rHSA).

Overview of B000 production process.

To purify rHSA from rice grain, rice grain expressing recombinant human serum, as described above, was dehusked, ground to flour and mixed for 30 min. with rHSA extraction buffer (Sodium Acetate, pH 4.9). After clarification by filtration, the filtrate was loaded onto an anion-exchange column (Q-Sepharose; GE Healthcare) at a rate of approximately 150 cm/hr. Once loaded, the resin was then washed with equilibration buffer to remove any unbound protein. The eluted rHSA is approximately 90% pure as analyzed by SDS-PAGE. Blue-Sepharose (GE Healthcare) can then be used to increase the purity from 90% to approximately 98% based on scanning analysis of SDS-PAGE gels. The rHSA is then concentrated and desalted via ultrafiltration prior to freeze drying.

Purified recombinant HSA was subjected to a range of biochemical and biophysical characterizations for comparison to pHSA (Plasma-derived HSA) (**Table E2**). These tests indicate that rHSA expressed in rice is equivalent to pHSA

Table E2

Properties of recombinant albumin (rHSA) as compared to plasma derived albumin (pHSA)

Property	pHSA	rHSA
N-terminal sequence	DAHKSE	DAHKSE
Glycosylation	None	None
SDS-PAGE/Western	~66 kDa	~66 kDa
Molecular mass (MALDI)	66.9-68.1 kDa	66.8-67.9 kDa
Isoelectric focusing point	pI 5.3	pI 5.3
Ligand binding	Yes	Yes
Thermal stability	Midpoint 65°C	Midpoint 65°C
Esterase activity	Yes	Yes
Protease sensitivity	Same as each other	Same as each other

B0000C Production Process: For Ventria grown rice, the rice was harvested by combine or by hand. During this process the mature seeds were separated from the vegetative plant matter by the combine separator or by manual labor. The harvested rice was dried to approximately 12% moisture at which point it is suitable for storage in a clean grain bin, storage tote, supersack, or other container that will protect the grain from birds, rodents, lizards, insects and other pests. When the rice grain is needed for flour, it is first dehusked or dehulled. This process is done under vacuum such that debris and the outer part of the seed are swept away from the endosperm and germ or bran layer. The dehusked grain is then either washed and dried, or washed and processed directly as in wet homogenization, or processed further in the dry, dehusked state. The dry, dehusked material may be debranned by a rice polishing or debranning machine which are common to white rice producers.

Debranned, dehusked rice may be washed at this point and wet-milled or dried for dry milling or processed directly by grinding into flour. Milling with the least amount of shear and heat is preferred as such with a roller mill or pin mill. A hammermill is also suitable. The flour should be ground such that the protein can be extracted to 90% in less than 5 minutes in water with hard agitation. Normally that requires a size of particle that is smaller than 400 micrometers or 4 mm. However, larger particles can be extracted if given longer time. Alternatively, the grain can be washed and wet milled with a liquid homogenizer set up such that 90% of the extractable protein is solubilized.

The flour slurry is typically mixed at a ratio of at least 3 parts water to 1 part flour and up to 20 parts water to 1 part flour. The water typically contains suitable buffers such as Tris/HCl. Citrate, Phosphate, HEPES, or the like, such that the pH is maintained around pH 7 and a small amount of salt such as 100 mM NaCl. After the slurry is homogenized in the case of wet milling, or mixed thoroughly for dry flour, the bulk solids are removed from the slurry by way of solid liquid separation. This is carried out by decanting, centrifugation, or filtration; for example using plate and frame with pads, pressure filter, belt filter, vacuum flask, hydroclone, or vacuum belt filter. After filtration, the compressed cake should be washed with extraction buffer to recover protein from the cake. The addition of diatomateous earth or other filter media is useful in promoting the clarity of the filtrate but is not necessary given the right equipment. Alternatively, a flocculating agent may be used to aid in clarification. The clarified filtrate should be checked for its albumin content and verified that the recovery is consistent with the determined expression level in the rice seed.

In order to remove starches, precipitable proteins, viruses, and other contaminants, 5 M acetic acid is added to the clarified filtrate until the pH reaches 5.0 and the solution turns white. The white solution is agitated for at least 20 minutes to encourage precipitation of insoluble materials. The precipitated solution is then filtered through a depth filter, such as a canister filter, cartridge filter or other filtration device to reach an optical clarity that is suitable for ultrafiltration, or less that 10 NTU (nephelometry turbidity units). It can also be clarified with a filter press, pressure filter, or alternatively by using a ceramic filter or other material that utilizes cross-flow. In addition, this material is suitable for direct application to an expanded bed chromatography column.

In a preferred method, the clarified filtrate is clarified via filtration through a 0.2 micron filter, and neutralized to pH 7.0 with 1M NaOH. This material is then suitable for ultrafiltration by hollow fiber, flat sheet, or spiral wound cross flow filtration. The material can be passed through a membrane of 100 kilodalton (kDa) size or larger to remove viruses, unwanted larger contaminants, and aggregates. The material that passes through the membrane can be concentrated by a 10 or 30 kDa crossflow membrane and then the same membrane can be used to prepare the solution for chromatography. The concentrated material can then diafiltered with column equilibration buffer until the conductivity and the pH are equalized.

The preferred buffer for anion exchange chromatography on GE DEAE sepharose or GE Q sepharose is 10 or 20 mM Tris/HCl buffer pH balanced to pH 8.0. In contrast, the preferred buffer for cation exchange, for example via the use of for negatively charged resins or

negatively charged resins mixed with a hydrophobic linker (mixed mode absorbents), or alternatively blue Cibicron such Blue Sepharose (GE) is acetate or citrate buffer pH balanced to 4.8 to 5.0

For either system the albumin and other similarly charged proteins will be retained by the matrix and washing is conducted to remove loosely bound material by washing with at least 5 column volumes of loading buffer, which may also include detergents as deemed necessary to help remove hydrophobic impurities. The material can be eluted by charging the column with the same or modified buffers with the pH increased 2-4 units for cation exchange or decreased 2-4 units for anion exchange. The resulting change in pH will allow for the exchange of ions and the protein will be eluted in a sharp band. To increase the purity of the elution fraction, the elution peak can be scrutinized such that the first portion (10%) or last (10%) or both portions can be excluded from the main elution peak. In the preferred method, a solution containing phosphate at 100 mM and pH adjusted to pH 4.0 including 10 mM NaCl is used to elute the protein from GE Q sepharose (Fast Flow). In this instance, pH and conductivity are used to elute the material allowing the discrimination between non-binding contaminants (flow through and wash) and tighter binding contaminants (those that are retained on the column in 100 mM Phosphate, 10 mM NaCl, and pH adjusted to 4.0).

After elution, if the pH of the eluted material has a pH of less than 6.0, then it is neutralized with 1M NaOH. The resulting solution is then diafiltered against the same buffer for the next chromatography step, which in a preferred method involves flowing the elutent through a column of the same matrix (i.e. Q sepharose) except in the non-binding mode with 100 mM Phosphate, 10 mM NaCl, and pH 7.0.

The second column step uses the same principles as the first but in reverse mode such that the contaminants that were co-eluted on the binding column have an opportunity to be retained on the matrix at a neutral pH. The flow through material from the first capture column can also be treated with a variety of alternative types of chromatography approaches, for example, cation exchange, hydrophobic, mixed mode, or gel filtration chromatography.

In a preferred method, the flow through material from the Q sepharose non-binding column is concentrated on a 10 kDa or 30 kDa crossflow membrane until the concentration is between 15 and 25% albumin. The buffer is then changed by diafiltration into a suitable buffer for cell culture such as Dulbeccos PBS or alternatively 20 mM Phosphate, 50 mM NaCl, and pH 7.0. The material is then sterile grade filtered into a sterile container. The sterile filtered material

may be treated with detergent to destroy enveloped viruses and to aid in the removal of hydrophobic toxins and contaminants. In a preferred method, 0.5% v/v Triton X-114 or X-100 is added to the 15 to 25% albumin solution at room temperature (less than 23 C and greater than 18 C) and the solution is agitated or stirred for at least hour. The material is then passed over a hydrophobic resin with a molecular weight exclusion limit that is much less than the molecular weight of albumin. Many commercially available resins are available including those from Biorad and Pall Corporation

The material that is passed over the column may then be tested in cells that are sensitive to detergent to confirm biological activity. The residual detergent that remains should typically be less than 0.005% with respect to the albumin solution. The detergent free flow through can then be sterile filtered into containers for direct shipment, or can have stabilizers added, or can be subjected to pasteurization with stabilizers, or can have stabilizers added before drying or dried directly. The material may be dried by lyophilization or spray drying. Prior to drying, in some instances, it may be useful to subject the material to a virus filtration step using a disposable, validated, virus removing capsule such as is available from GE, Pall, and Millipore. It is common in the art to understand that a prefiltration step may be necessary in order to effectively and economically pass the concentrated material through a 20 nm filter.

Results: Rice flour was extracted at 1:5 ratio in phosphate buffered saline and mixed for 20 minutes. The liquid was clarified using a Nalgene filter flask The subsequent clarified extract was subjected to acid precipitation as is described in the methods. The solution was then filtered and neutralized to give a clarified filtrate. This material was diafiltered against 50 mM Tris/Cl pH 8.0 until the material and buffer were equilibrated. The material was then loaded (300-600 cmh) on a pre-equilibrated GE Q-sepharose column to allow for 50 g/L binding capacity The loaded material was washed with the same buffer and the material was then eluted with 100 mM Phosphate, 10 mM NaCl, and pH 4.0 as described above. The material eluted in a sharp peak and the collected eluate had a stable pH of about 5.8. Albumin produced using this method was compared to other sources of Albumin as more fully disclosed below. The eluate was collected in a pool and 1M NaOH was added until the pH was greater than 6.0. The material was then concentrated on a 10 kDa regenerated cellulose membrane approximately 5 fold and approximately five equal volume diafiltrations were carried out with 100 mM phosphate, 10 mM NaCl, pH 7.0. The final diafiltered material was checked for albumin protein content (in relation to the expression level in the starting material should be greater than 80%) and endotoxin level (should be less than 100 EU/mg depending on the feed material). This material was passed (60-160 cmh) over a Q-sepharose column, equilibrated with 100 mM

phosphate, 10 mM NaCl, pH 7.0, of sufficient size to allow for approximately 2-3 times loading volume. The material was washed through the resin with the same buffer and collected. The collected material was diafiltered on a 10 kDa regenerated cellulose membrane and concentrated approximately 10 fold or until the albumin concentration reaches at least 10% or not more than 20% and five equal volume diafiltrations were performed with 20 mM phosphate, 50 mM NaCl, pH 7.0. After sterile grade filtration (0.2 µm), the solution was agitated for 1 hour with 0.5% (v/v) Triton X -100 at 20 +/- 2°C. After the incubation, the material was passed through Pall SDR resin according to the manufacturer's directions The flow through material was sterile grade filtered into sterile containers and refrigerated or freeze dried as is common for protein and salt solutions.

Example 6: Comparison of recombinant albumin produced from rice using B0000C process compared to other sources of albumin and previous methods for the production of Albumin.

Methods: Albumin prepared using process B000C described in Example 5, was compared to albumin prepared using an alternative process (B000) which was previously used to prepare recombinant albumin (Cellastim (Batches B202 to B217)).

Albumin production (old process, B000):

Rice flour and 25 mM Sodium phosphate, 50 mM Sodium Chloride, was pH balanced to 6.5 with NaOH and mixed for 20 minutes at room temperature with a S/L ratio of approximately 1.10. Filter aid (Cellpure 300) was added at 10 g/L and the slurry was filtered by filter press, vacuum filtration, or centrifugation. The clarified filtrate was acid precipitated to about pH 5.0 with 1 M acetic acid. The resulting solution was filtered as described above with the addition of 5 g/L filter aid (Cellpure 300). The material was neutralized immediately to pH 6.5 to 7.0 with 1M NaOH. The material was diafiltered (10 kDa regenerated cellulose for all UFDF steps) with 5 equal diavolumes of the same buffer used for extraction. The material was loaded on a pre equilibrated Q-sepharose column (GE Healthcare) to allow for 8 g albumin binding per liter of resin at 60 cmh.

After washing the column with 5 column volumes of the same buffer, the albumin was eluted by increasing the salt concentration to 250 mM NaCl in one step. The resulting material was diafiltered against 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0 with 5-7 equal diavolumes. The resulting material was passed over a Q-sepharose column equilibrated with the 100 mM Sodium Phosphate, 10 mM NaCl, pH 7.0, and collected as flow-through. The flow-through

material was then concentrated and diafiltered against 20 mM sodium phosphate, 10 mM NaCl, pH 7.0 with 5 diavolumes. The final concentrated material was sterile filtered and incubated with 10 g/L of the detergent CHAPS ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid) and mixed at room temperature for 1 hour. After the one hour incubation, the material was passed over a Biorad SM-2 column. The material was sterile filtered and freeze dried.

Size Exclusion Chromatography analysis.

Purity analysis by HPLC was carried out in 100 mM phosphate, pH 7.0 on a GF-250 column (Agilent Technologies) at a flow rate of 1 ml/min with the detector set at 214 and 280 nm. A standard curve was developed by injecting 5 different dilutions made by dry powder with a correction factor of 0.92 for salt and moisture. The main peak from 214 nm was integrated either by retention time or alternatively baseline The unknown sample was injected at a concentration that is within the range of the standard injections. The unknown concentration of albumin per dry powder weight (purity) was calculated from the standard curve. In a typical experiment, the 0, 5, 8, 10, 15, and 20 µg of the standard was injected followed by approximately 10 µg of unknown sample in approximately 50 µL injection volume. The correlation coefficient for the standard curve after integrating the peaks was typically above 0.98.

SDS PAGE and Densitometry: (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Samples were prepared by diluting the protein solutions to 1-2 mg/ml to enable a defined amount of each protein to be loaded on to each well. The sample was mixed 1:1 with Tris-Glycine SDS sample buffer (LC2673 Novex) containing reducing agent (Invitrogen NP0004) and heated to 70 °C for 5 minutes. The sample was loaded (10, 20, or 30 µg) onto a Novex 4-20% precast gel and separated at constant voltage (130V) in standard Tris-Glycine-SDS running buffer. The electrophoresis was ended when the tracking dye reached the end of the gel. A molecular weight marker was included in the first lane as a reference.

The gel was stained with G Bioscience (786-35G) and destained with water A digital image was obtained with a Hewlett Packard Scanner (G4010). The image file was then opened with UN-SCAN-IT (Silk Scientific Corp.). The densitometry was carried out with positive image analysis in 256 grayscale in which all visible bands were included as individual segments. The background noise was corrected by four corner interpolation as specified in the software for each segment. The signal for each segment or band was then calculated from the product of the # of pixels and the average pixel intensity (0-255). The sum of the signals for an entire lane (all visible segments or bands) was taken as 100% and the impurity bands were subtracted to

calculate the albumin purity. The percent of each contaminating protein in each band was calculated as the number of peptides identified for that contaminant protein as determined by peptide mapping divided by the total number of all peptides identified in a particular band. The image analysis was repeated 3 times such that the standard deviation is less than 0.5% out of 100%

Determination of endotoxin by the Pyrogene rFC method.

Endotoxin content was determined by the Pyrogene rFC method Lyophilized endotoxin standard was mixed with endotoxin free water as specified by the manufacturer (Lonza) to develop a standard curve. The protein samples were either diluted as is for liquid or alternatively, reconstituted with endotoxin free water for powder. Different dilutions were prepared such that the readings should appear within the range of the standard curve. The samples were heated to 100 °C for 10 minutes to dissociate unwanted molecular interactions. In a typical experiment, the sample and standard were added at 100 µl per well, with 0, 0.001, 0.005, 0.01, 0.05, and 0.1 endotoxin units per well. The samples were also added at 100 µl and extra samples were included such that spiking with 0.001 -0 01 endotoxin units per well were added to test for assay inhibition or interference. The working reagent was prepared according to the manufacturer (Lonza) by mixing the rFC enzyme, assay buffer, and substrate in a 1:4:5 ratio, respectively. The working reagent was added to the wells at equal volume to the sample or standards. The fluorescence plate reader (Biotek FLX 800T) was set for excitation at 380 nm (bandwidth =20 nm) and the emission wavelength was set at 440 nm (bandwidth =30 nm) The reading taken at time zero is subtracted from the reading taken after 1 hour at 37 °C. The readings were considered valid if the correlation coefficient, slope, and Y-intercept for the standards was within the set limits, and the spiking experiments show that the spiked endotoxin was measureable and recoverable within the set limits In addition, the standard deviation for duplicate samples should be in reasonable agreement such that the standard deviation was within a specified arbitrarily chosen limit. All samples were collected aseptically and the tubes/vials/containers used for testing were verified to be extremely low endotoxin following good laboratory practices as they relate to accurate and precise endotoxin testing.

Determination of Cell Viability

The hybridoma cell line AE1 (ATCC) was maintained in DMEM basic media containing 5% fetal bovine serum (FBS). Albumin was tested under serum-free conditions (AFM6, KC Bio, Kansas) without supplementation of fetal bovine serum. The cells were subculture from 5% FBS to serum free media over multiple passages. At each subculture, the cells were analyzed for total cell count and viability in the presence of the indicated concentrations of albumin (As assessed

by trypsinization and direct counting using a Neubauer haemocytometer). The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Date show the number of viable cells / ml divided by 10^5.

Determination of Detergent

The detergent concentration for the albumin was determined by a detergent (cell based) assay. Briefly, detergent sensitive cells were spiked with different amounts of detergent and the resulting cell viability cell determination used to generate a standard curve consisting of 16 independent data points. The change in viability with respect to the change in detergent concentration was plotted and fitted with a logarithmic function. This equation was then used to calculate the unknown detergent concentrations in samples tested in the same cell based assay. The correlation coefficient for the standard curve for the data given was 0.9816. Typically detergent concentrations of greater than about 10 ppm per Cellastim dry weight, result in noticeable toxic activity. By comparison in a 10% albumin solution, toxic effects of detergent become apparent when the detergent concentration is above about 100 ppm to 200 ppm or 0.01% to 0.02 % (v/v).

Results & Discussion:

I. Analysis by Size Exclusion chromatography of plasma derived serum (Sigma Albumin), and recombinant HSA (Cellastim) produced using the new process (B000C) (Cellastim P0171) and the old process (Cellastim P0107).

The HPLC size exclusion profiles (**FIG. 8A, C & D**) for the three types of albumin show that in terms of overall purity the different albumin preparations are generally similar. Specifically, the peaks at around 4.5 kDa and 240 kDa are the internal controls, while all three products contain a very small amount of an off main peak signal at about 10-12 kDa.

Human plasma derived albumin (Sigma Albumin) (**FIG. 8A**), contains a contaminant at around 17 kDa, by comparison the recombinant rice derived albumin using the new process (Cellastim P0171) (**FIG. 8C**) contains two protein contaminants of around 44 KDa and 55 kDa that occur in Cellastim made with the new **FIG** at significantly higher levels than when using the old process (Cellastim P0107) (**FIG. 8B**). These peaks are not completely resolved in the HPLC separations, but can be seen as more clearly in the overlaid profiles of Cellastim P0171 and Sigma albumin (**FIG. 8D**) and Cellastim made using the old and new processes shown in **FIG. 8E.**

The proteins corresponding to these peaks represent about 5% of all of the contaminant proteins identified by Peptide Mass Fingerprinting analysis of the main albumin peak in Cellastim produced using the process described in Example 5, as discussed further below

All albumin products tested also contained a peak at around 130 kDa that most likely represents albumin dimers, it is noticeable that the Cellastim dimer peak is significantly smaller than the plasma derived albumin The creation of aggregated albumin is an indicator of protein degradation which is used as one marker for degradation or loss of stability industry wide. It is likely that the Hsps present in Cellastim promote the disaggregation of the albumin, therefore reducing the number of dimers, since it is a commonly known function of Hsp 70 and other Hsp proteins

II Analysis by SDS PAGE of Cellastim batch **P0171** (New process) compared to albumin produced by Millipore/Novozymes (Cat No. 9301-01)

Results: SDS-PAGE analysis (**FIG. 9A & FIG. 9B**) shows that in terms of overall purity the products are generally similar. **FIG. 9A** shows a comparison of Cellastim P0171 and Cellprime albumin (Millipore/Novozymes). Lane 1 is the molecular weight marker. Lane 4 is the Cellastim albumin (10 µg) and Lane 7 is the Cellprime albumin (10 µg). **FIG. 9B** shows a comparison by SDS PAGE analysis of three Cellastim lots from the previous process (B000) (Lane 2, 3, and 4), and the new Cellastim Process (B0000C) (Lane 6, 7, and 8). The six samples were loaded at 20 µg per lane.

Visual inspection of the gel shows that the new process which meets more rigorous specifications is more consistent among the 3 lots tested. (**FIG. 9B**, lane 2, 3, 4 vs. lane 6, 7, 8). The banding pattern is significantly different among the three samples from the previous process as compared to the new process. Importantly, the new process samples have significantly less aggregates at around 250 KDa than the old process samples have. (Average greater than 2 % for the old process, and average less than 1 % for the new process). The identity of the protein contaminates was that are enriched in Cellastim produced using the new process is discussed further below.

III. Analysis of endotoxin, detergent and growth promoting abilities of old and new batches of Cellastim. A comparison of the performance of the two different processes for preparing several different lots of albumin (**Tables E3 and E4**) demonstrates that the old

process produced recombinant albumin that contained significantly more endotoxin, and detergent compared to the new process described in Example 5, and resulted in a product that significantly enhanced cell viability.

Table E3

Cellastim - Old Process B000

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					1mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/27/08	B202	70.6	25.3	815 3	15	7.9	4.1	0.9
2/27/08	B203	58.8	23.1	267.7	15.8	12.4	6.5	4.1
2/28/08	B204	59.9	29.2	589.2	16.3	11 1	5.2	2.9
2/28/08	B205	63	64.3	133.4	14	11.6	6.9	3.6
3/3/08	B206	41.1	35.1	1182.2	14.9	2 3	0.0	0.0
3/3/08	B207	94.2	28.7	388	16.5	12.7	7.6	4
3/4/08	B208	24.5	>80	467.2	15 3	11.4	7.1	2.7
3/4/08	B209	66.1	91.8	406.4	16.7	15.2	6.9	4.1
3/4/08	B210	87.3	67 8	76.4	17.5	19.1	15.4	8.5
3/11/08	B217	62.09	4.4	127.3	16.7	18.7	13.2	6.6
Averages			**44.97**	**445.31**	**15.87**	**12.24**	**7.29**	**3.76**

Table E4

Cellastim - New Process B0000C

Production Date	Batch Number	Grams product	EU/mg Dry Material	Detergent (ppm)	Viability (number of viable cells / ml / 10^5)			
					0.5mg/ ml	2 mg/ml	5 mg / ml	10 /mg ml
2/2/2009	B0032C	209 4	0 35	3 1	13.1	15.7	13.4	11 2
2/2/2009	B0033C	271.4	0.26	7.9	18.4	17.4	15.1	14.3
2/10/2009	B0041C	247.1	0.18	5.4	15.4	17 2	14.1	12.1
4/27/2009	B0118C	617.4	0.11	Less than 1	15.7	15.4	19.2	16.8
5/14/2009	B0138C	610 6	0 48	Less than 1	12.5	15.8	15.1	14.6
6/9/2009	B0158C	598.0	0.13	3.9	13.7	17.1	13.2	11
6/15/2009	B0162C	618.6	0.20	2.0	15.5	17 5	17.2	14.7

7/28/2009	B0196C	507.0	0.36	3.9	17.4	17.3	18.8	14.8
8/26/2009	B0219C	851.4	0.86	8.2	17.9	16.3	16.6	13.7
8/31/2009	B0220C	897.8	0.93	Less than 1	13.5	16	15.5	15.1
9/9/2009	B0227C	929.9	0.13	Less than 1	11.1	14.4	12.5	11.4
Averages			**0.36**	**3.45**	**14.92**	**16.37**	**15.52**	**13.6**

Discussion: Re-engineering the old process to create the new process described in Example 5 resulted in significant changes in both overall product purity, and performance, as described more fully below.

The changes made it possible to make products that were lower in detergent, lower in endotoxin, and increased purity. Specifically, the new process routinely produced recombinant albumin with an overall purity of greater than about 95%. By comparison the old method routinely produced albumin with a maximum purity of about 90 %. Surprisingly, despite the increased product purity, these changes in processing also resulted in enhanced co-purification of heat shock proteins, (see below) with the recombinant albumin. Without being bound by any particular theory of operation, it is believed that the combination of high albumin purity, relative lack of endotoxin and / or detergent, and co-purification of heat shock proteins results in a product that significantly out performs previous methods for preparing albumin.

Specifically **Tables E3** and **E4** demonstrate that the new process for producing recombinant albumin results in a product that, for example at 5 mg /ml, results in an average batch to batch 100 percent improvement in cell viability (at 5 mg /ml), and also results in a product with an average 100-fold less endotoxin, and 100 fold less detergent than the old process.

Example 7: Analysis of the effects on cell growth and viability.

To compare the cell growth promoting abilities of the recombinant albumin prepared above, to other commercially available albumin products, the different sources of albumin they were compared side by side in a cell growth and viability assay. The three products tested were (Cellastim, Lot # P0153) Celiprime albumin (Millipore/Novozymes Cat No. #9301-01), and plasma derived albumin (Seracare Cat No. #HS-400-60).

Methods: Specially conditioned Hybridoma cells AE1 were seeded in DF12/ITSE at a density of 0.5×10^5 cells per ml of media after washing twice with same media to remove residual media.

The media and cells were then left untreated (negative control), treated with Seracare albumin, treated with Cellprime albumin, and treated with rice derived recombinant albumin at the concentrations shown in the figure legend. The cells were grown under standard culture conditions (5% CO_2 and 37 °C) for approximately 70 hours after which the viability for the cultures was measured. The experiments were conducted in duplicate. Results are shown in FIG. 10A-FIG. 10C.

Results: Novazyme's Cellprime albumin caused a loss in viability (cross-hatch bars). Seracare albumin (white bars) caused a measureable increase in viability but not as large an increase as is seen with recombinant albumin made in rice with the new process (Cellastim) (black bars). Under these conditions the rice derived recombinant albumin was approximately 5 times as active in promoting cell viability compared to any of the other albumin products, at any concentration tested. The negative control is represented by the striped bars.

Discussion: Given the possibility that the other commercially available albumin products may have similar overall purity, endotoxin and detergent levels to rice derived recombinant albumin, the dramatically superior performance of the recombinant rice derived albumin compared to other commercially available albumins suggests that the previously un-identified protein contaminants identified in Cellastim compared to the plasma derived albumin (Example 5) could be having a positive impact on cell viability. To identify and then characterize the impact of these proteins on the properties of Cellastim, a sample of the recombinant albumin was subjected to peptide mass finger printing, as described below.

Example 8: Peptide Mass Finger printing of recombinant human serum albumin.

Methods: Samples of albumin were analyzed to determine significant protein contaminants using a NanoLCMS/MS peptide sequencing system (ProtTech, Inc.), and proprietary software to identify the proteins based on the molecular weight of the peptide fragments. In brief, samples of albumin were analyzed by SDS-PAGE, and each major band gel band was destained, cleaned, and digested in-gel with sequencing grade modified trypsin. The resulting peptide mixture was analyzed by a LC-MS/MS system, in which a high pressure liquid chromatography (HPLC) with a 75 micrometer inner diameter reverse phase C18 column was used in-line coupled with an ion trap mass spectrometer. The mass spectrometric data acquired was used to search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting

Results: Upon testing of three representative lots of recombinant albumin, three Hsp70 proteins were identified by Peptide Mass Fingerprinting (**Table E5**) The three specific sequences identified: ABF95267, ABA97211, and BAD 07938 were compared to the non redundant database to identify highly related and homologous proteins. The results of the top hits from each of these comparisons is shown in **Tables E6, E7 and E8** below.

Table E5

Peptides identified from Cellastim by mass finger printing

Sequence	Peptide
ABF95267	ATAGDTHLGGEDFDNRVVPGPADKSPMIVVTYKGEEK NAVITVPAYFN DSQRIINEPTAAAIAYGLDKK (**SEQ. ID. No. 2**)
AAB63469 BAD07938 BAD07713	NQAAVNPER NGHVFIIANDQGNRIVNKDGKPYIQVK IINEPTAAAIAYGLDKK KLGTVIGIDLGTTYSCVGVYK VEIESLFDGTDSFSEPLTR (**SEQ. ID. No. 3**)
ABA97211	NQADSVVYQTEK KQDITITGASTLPKDEVERDVVLLDVTPLSLSLGLET LGGVMTK (**SEQ. ID. No. 4**)

Results of sequence comparisons to ABF95267 sequences in the non redundant database of protein sequences in Genbank® (Nucleic Acids Research, 2008 Jan;36(Database issue):D25-30) are shown in **Table E6** below.

Table E6 Sequences producing significant alignments with ABF95267:			
Gene Refs	Gene description	(Bits)	Value
ref|NP_001140835.1|	hypothetical protein LOC100272911 [Zea ma..	79.7	8e-14
ref|XP_002465468.1|	hypothetical protein SORBIDRAFT_01g039390..	79.7	9e-14
ref|NP_001049719.1|	Os03g0277300 [Oryza sativa (japonica cult..	79.7	9e-14
gb|ACJ54890.1|	heat shock protein 70 [Oryza sativa Japonica G...	79.7	9e-14
Sp|P09189.1|	HSP7C_PETHY RecName: Full=Heat shock cognate 70 k...	77.0	5e-13
emb|CAA31663.1|	hsp70 (AA 6 - 651) [Petunia x hybrida]	77.0	5e-13
ref|XP_002312089.1|	predicted protein [Populus trichocarpa] >..	77.0	5e-13
sp|P24629.1|	HSP71_SOLLC RecName: Full=Heat shock cognate 70 k.	77.0	5e-13
gb|AAB99745.1|	HSP70 [Triticum aestivum]	76.6	6e-13
gb|AAB42159.1|	Hsc70 [Lycopersicon esculentum]	76.6	7e-13
gb|ACD45076.1|	heat-shock protein 70 [Dactylis glomerata]	76.3	8e-13
ref|XP_002512741.1	heat shock protein, putative [Ricinus com...	75.9	1e-12
ref|XP_002512742.1|	heat shock protein, putative [Ricinus com..	75.9	1e-12
gb|AAA82975.1|	PsHSP71.2 >emb|CAA67867.1| heat shock protein ...	75.9	1e-12
gb|AAS09825.1|	heat shock cognate protein 70 [Thellungiella h..	75.9	1e-12
emb|CAA44820.1|	heat shock protein 70 [Nicotiana tabacum]	75.5	2e-12
ref|NP_001055754.1|	Os05g0460000 [Oryza sativa (japonica cult...	75.5	2e-12
ref|NP_001051724.1|	Os03g0821100 [Oryza sativa (japonica cult...	75.1	2e-12
ref|XP_002456611.1|	hypothetical protein SORBIDRAFT_03g039360..	75.1	2e-12
ref|NP_001044757.1|	Os01g0840100 [Oryza sativa (japonica cult...	75.1	2e-12
gb|ACR35910.1|	unknown [Zea mays]	75.1	2e-12
ref|XP_002284017.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002532297.1|	heat shock protein, putative [Ricinus com..	75.1	2e-12
ref|XP_002284008.1|	PREDICTED: similar to HSC70-1 (heat shock...	75.1	2e-12
ref|XP_002283532.1|	PREDICTED: similar to HSC70-1 (heat shock..	74.7	3e-12
ref|XP_002332067.1|	predicted protein [Populus trichocarpa	74.7	3e-12
gb|AAF34134.1|	high molecular weight heat shock protein [Malu..	74.7	3e-12
gb|EEC76425.1|	hypothetical protein OsI_14101 [Oryza sativa I...	74.7	3e-12
ref|XP_002316294.1|	predicted protein [Populus trichocarpa] >..	74.7	3e-12
ref|XP_002283516.1|	PREDICTED: similar to HSC70-1 (heat shock..	74.3	3e-12
ref|XP_002441219.1|	hypothetical protein SORBIDRAFT_09g022580..	74.3	4e-12

Results of sequence comparisons of ABB63469 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E7** below:

Table E7 Sequences producing significant alignments with AAB63469:			
Gene Refs	Gene description	(Bits)	Value
emb|CAP31983.1|	C. briggsae CBR-HSP-4 protein [Caenorhabditis..	50.1	7e-05
db|BAG60366.1|	unnamed protein product [Homo sapiens]	49.7	8e-05
ref|YP_002421952.1|	chaperone protein DnaK [Methylobacterium . .	49.7	9e-05
ref|YP_001640420.1|	chaperone protein DnaK [Methylobacterium . .	49.7	9e-05
ref|NP_001105893.1|	Binding protein homolog1 precursor [Zea m...	49.3	1e-04
gb|AAA62325.1|	HSP70	49.3	1e-04
ref|YP_001756576.1|	chaperone protein DnaK [Methylobacterium ...	49.3	1e-04
gb|AAB63469.1|	endosperm lumenal binding protein [Oryza sativa]	49.3	1e-04
ref|YP_001925829.1|	chaperone protein DnaK [Methylobacterium .	49.3	1e-04
ref|NP_001105894.1|	Binding protein homolog2 precursor [Zea m...	49.3	1e-04
gb|ACF86491.1|	unknown [Zea mays]	49.3	1e-04
ref|NP_001045675.1|	Os02g0115900 [Oryza sativa (japonica cult. .	49.3	1e-04
ref|ZP_02191025.1|	Molecular chaperone [alpha proteobacterium..	49.3	1e-04
ref|XP_001701685.1|	binding protein 1 [Chlamydomonas reinhard...	48.5	2e-04
ref|XP_001701884.1|	binding protein 2 [Chlamydomonas reinhard..	48.5	2e-04
emb|CAC37635.1|	luminal binding protein, BiP [Scherffelia dubia]	48.1	3e-04
gb|AAM93256.1|	heat shock protein 70-C [Heterodera glycines] ...	48.1	3e-04

Results of sequence comparisons of sequence ABA97211 to sequences inthe non redundant database of protein sequences in GenBank® are shown in **Table E8** below:

Table E8 Sequences producing significant alignments with ABA97211			
Gene Refs	Gene description	(Bits)	Value
gb|AAK13022.1|	heat shock protein 70 [Fibrobacter succinogene...	44.3	0.004
ref|XP_002442079.1|	hypothetical protein SORBIDRAFT_08g009580...	44.3	0.004
gb|EEC69073.1|	hypothetical protein OsI_37938 [Oryza sativa I. .	44.3	0.004
ref|XP_001752769.1|	predicted protein [Physcomitrella patens ...	44.3	0.004
ref|NP_001066486.1|	Os12g0244100 [Oryza sativa (japonica cult...	44.3	0.004
gb|ACT65562.1|	70 kDa heat shock protein [Triticum aestivum]	43.9	0.005
ref|NP_001152528.1|	stromal 70 kDa heat shock-related protein...	43.9	0.005
gb|ACN31310.1|	unknown [Zea mays]	43.9	0.005
ref|XP_001772650.1|	predicted protein [Physcomitrella patens ...	43.9	0.005
ref|NP_001146752.1|	hypothetical protein LOC100280354 [Zea ma ..	43.9	0.005

gb|ABP65327.1|	chloroplast heat shock protein 70 [Pennisetum ...	43.9	0.005
gb|AAO72585.1|	heat shock-related protein [Oryza sativa (japo...	43.5	0.007
ref|YP_001740846.1|	Chaperone protein dnaK (Heat shock protei...	43.5	0.008
ref|ZP_03728467.1|	chaperone protein DnaK [Dethiobacter alkal ...	43.1	0.009

Discussion: Peptide Mass Fingerprinting identified 3 rice heat shock protein super family members that co-purify with albumin, 2 Rice HSP70 genes, (gb|ACJ54890.1|), EEC69073, and AAB63469 - a BiP homolog from rice endosperm tissue (endosperm lumenal binding protein). The complete amino acid sequences coded by these genes are listed below:

Gene gb|ACJ54890.1| heat shock protein 70 [Oryza sativa Japonica Group] HSP70 was found to occur in recombinant albumin in Cellastim at approximately 0.07% wt / wt. Its complete amino acid coding sequence (**SEQ. ID. No. 5**) is provided below:

```
MAGNKGEGPA IGIDLGTTYS CVGVWQHDRV EIIANDQGNR TTPSYVAFTD TERLIGDAAK
NQVAMNPTNT VFDAKRLIGR RFSDPSVQAD MKMWPFKVVP GPADKPMIVV TYKGEEKKFS
121 AEEISSMVLT KMKEIAEAFL STTIKNAVIT VPAYFNDSQR QATKDAGVIS GLNVMRIINE
181 PTAAAIAYGL DKKAASTGEK NVLIFDLGGG TFDVSILTIE EGIFEVKATA GDTHLGGEDF
241 DNRMVNHFVQ EFKRKHKKDI TGNPRALRRL RTACERAKRT LSSTAQTTIE IESLYEGIDF
301 YATITRARFE ELNMDLFRRC MEPVEKCLRD AKMDKAQIHD VVLVGGSTRI PKVQQLLQDF
361 FNGKELCKSI NPDEAVAYGA AVQAAILSGE GNQRVQDLLL LDVTPLSLGL ETAGGVMTVL
421 IPRNTTIPTK KEQVFSTYSD NQPGVLIQVY EGERTRTKDN NLLGKFELTG IPPAPRGVPQ
481 INVTFDIDAN GILNVSAEDK TTGKKNKITI TNDKGRLSKE EIERMVQEAE KYKAEDEQVR
541 HKVEARNALE NYAYNMRNTV RDEKIASKLP ADDKKKIEDA IEDAIKWLDG NQLAEADEFE
601 DKMKELESLC NPIISKMYQG GAGGPAGMDE DAPNGSAGTG GGSGAGPKIE EVD
```

AAB63469 BiP homolog from rice endosperm tissue (endosperm lumenal binding protein [Oryza sativa]) BiP was found to occur in recombinant albumin in Cellastim at about 0.09% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 6**) is provided below:

```
mdrvrgsafl lgvllagslf afsvakeetk klgtvigidl gttyscvgvy knghveiian
dqgnritpsw vaftdserli geaaknqaav npertifdvk rdigrkfeek evqrdmklvp
121 ykivnkigkp yiqvkikdge nkvfspeevs amilgkmket aeaylgkkin davvtvpayf
181 ndaqrqatkd agviaglnva riineptaaa iaygldkkgg eknilvfdlg ggtfdvsilt
241 idngvfevla tngdthlgge dfdqrimeyf iklikkkysk diskdnralg klrreaerak
301 ralsnqhqvr veieslfdgt dfsepitrar feelnndlfr ktmgpvkkam ddagleksqi
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361 heivlvggst ripkvqqilr dyfegkepnk gvnpdeavay gaavqgsils geggdetkdi
421 lildvapltl gietvggvmt kliprntvip tkksqvftty qdqqttvsiq vfegersmtk
481 dcrllgkfdl sgipaaprgt pqievtfevd angilnvkae dkgtgkseki titnekgrls
541 qeeidrmvre aeefaeedkk vkeridarnq letyvynmkn tvgdkdklad kleseekekv
601 eealkealew ldenqtaeke eyeeklkeve avcnpiisav yqrtggapgg rrrgrlddeh
661 del
```

EEC69073 / Osi_37938 [Oryza sativa Indica Group] The stromal HSP70 was found to occur in recombinant albumin in Cellastim at about 0.06% wt/ wt. Its complete amino acid coding sequence (**SEQ. ID. No. 7**) is provided below:

```
masftsqlga macgaapsts plaarrsgql fvgrkpaaas vqmrvpragr argvamrvac
61 ekvvgidlgt tnsavaameg gkptvitnae ggrttpsvva ytkggerlvg qiakrqavvn
121 pentffsvkr figrkmaevd deakqvsyhv vrddngnvkl dcpaigkqfa aeeisaqvlr
181 klvddaskfl ndkitkavvt vpayfndsqr tatkdagria glevlriine ptaaslaygf
241 ekknnetilv fdlgggtfdv svlevgdgvf evlstsgdth lggddfdkfy fcwvfyfgam
301 thetpkvvdw lasnfkkdeg idllkdkqal qrlteaaeka kmelstlsqt nislpfitat
361 adgpkhiett lsrakfeelc sdlidrlktp vtnalrdakl svdnldevil vggstripsv
421 qelvkkitgk dpnvtvnpde vvslgaavqg gvlagdvkdv vlldvtplsl gletlggvmt
481 kiiprnttlp tsksevfsta adgqtsvein vlqgerefvr dnkslgsfrl dgippaprgv
541 pqievkfdid angilsvaai dkgtgkkqdi titgastlpk devermveea dkfaqedkek
601 rdaidtknqa dsvvyqtekq lkelgdkvpa pvkekvdaki nelkealagg stqsmkdama
661 alneevmqig qamynqqpna gaagptpgad agptssggkg pndgdvidad ftdsn
```

Because these proteins only occur at low levels in the new batches of rice recombinant albumin relative to albumin, a pre-requisite for confirming that these contaminants are actually responsible for the superior growth promoting effects of the new batches of recombinant albumin is to determine whether the addition back of these components to albumin restores or enhances the growth promoting activities of the recombinant albumin at levels which are comparable to those actually identified for each component in Cellastim.

Example 9: Separation of heat shock proteins from recombinant albumin by affinity chromatography.

Methods: Cellastim produced using the new process [Lots P0153, P0156, and or P0171] powder was mixed with purified water at approximately 20 g/L. The resulting solution was diafiltered against 50 mM Tris/Cl, pH 7.0 with at least 5 equal volumes of buffer. The resulting solution was passed over an ATP agarose column and the resulting flow through was labeled as fraction A. The column was washed with 5 column volumes of the equilibration buffer and the

material bound to the ATP-agarose was eluted with 50 mM Tris/Cl, 1M KCl, pH7.0. The eluted material was labeled as fraction B. The wash was kept as fraction C. Fraction A was directly concentrated to 100 g/L and diafiltered with d-PBS. Fraction B was concentrated significantly, up to 20 fold or 100 fold in 50 mM Tris/Cl for further analysis. The wash fraction C was kept for further reference. For Western blotting, 10 µg of each protein fraction (by A280, where the e.c. (extinction coefficient) of albumin is 0.53 cm^2/mg and e.c. of Hsp70 is 0.41 cm^2/mg) were loaded on a 4-20% SDS PAGE gel in 2X SDS loading buffer. The samples were heated to 80 °C for approximately 5 minutes before loading. The separation was done at 200V (constant voltage) and ran for approximately 90 minutes. The resulting gel was rinsed in water for 30 minutes to 2 hours and then the proteins were transferred to a Nitrocellulose membrane at 30 mA (constant current) for 2 hours. The resulting blot contained the molecular weight marker proteins as a transfer control and was then blocked in 5% (w/v) milk powder in water. The primary monoclonal antibody (a mouse anti-bovine Hsp70(Sigma/Aldrich #H5147)) was added in 5% milk solution to the blot (1:2500) and the blot was incubated on a rocker with gentle rocking overnight at 4 °C. The blot was then washed 4 times for 10 minutes each in TDN and the secondary antibody (Pierce anti-mouse HRP conjugated) in 5% milk solution which was added at a dilution of 1:2500. After incubation at 4 °C for 2 to 3 hours, the blot was washed 4 times with TDN for 10 minutes each. The resulting blot was then incubated with pico (Pierce) chemiluminescent substrate for 5 minutes. Kodak photographic film was exposed to the blot in a dark room and the subsequent film was developed, rinsed, fixed, rinsed, and dried. To determine accurate transfer of the molecular weight marker position onto the film, a light emitting label was used.

Results: The results are shown in **FIG. 11**. The Western blot pictured shows that the separation scheme produces two populations of proteins in the A (flow through) and B (ATP binding) fractions. The starting material, (lane 2) the fraction A flow through, (lane 3) fraction C wash, (lane 4) and fraction B (lane 5) were tested for the ability to react to the monoclonal antibody. In addition, a commercially available Hsp70 protein that serves as a positive control was loaded in the last lane (lane 10). As shown in the blot in **FIG. 11**, the flow through fraction A (lane 3) does not contain significant amounts of Hsp70. The eluted and concentrated fraction B (lane 4) is highly reactive to the antibody as shown in the blot and indicates at least two distinct bands centered around the 75 kDa molecular weight marker. The wash fraction C (lane 5), indicates the presence of two bands that run at slightly below 75 kDa. In a separate independent experiment, the flow through fraction A (lane 7) again is not reactive to the antibody, and the wash fraction C (lane 8) is also not reactive to the antibody, but the fraction enriched in ATP binding proteins (Fraction B) shown in lane 9 gives the same banding pattern as was seen from the first separation.

Discussion: A separation protocol was developed to separate HSP70 proteins based on their ability to bind to ATP agarose affinity resin, and. was tested for its effectiveness. The procedure involved only minimal sample manipulation, using only ATP agarose, and ultrafiltration to concentrate and conduct buffer changes, and an anti-hsp70 antibody to detect the presence of hsps. The results of the procedure (**FIG. 11**) clearly demonstrates that while in 10 µg of starting material, 10 µg of flow through, or 10 µg of wash fraction there is insufficient hsp70 to be detected by the ant-Hsp antibody. By contrast, the fraction eluted from the ATP agarose column, contains at least two proteins that are clearly recognized by the anti-Hsp70 antibody. The results therefore show that the separation scheme was successful and reproducible on two independent chromatography runs and diafiltrations. Furthermore, the data substantiates the identification of heat shock proteins made by Peptide Mass Finger printing, as discussed earlier, and demonstrates that these proteins are functional and can be readily isolated and enriched by simple ATP agarose chromatography followed with diafiltration. It is concluded that the heat shock proteins co-purify with the recombinant albumin. Such co-purification is consistent with the hypothesis that the heat shock proteins are bound to the albumin, and that the albumin acts to stabilize the heat shock proteins in a stable conformation. Surprisingly, the recombinant albumin / heat shock protein complex retains significant ATPase activity (data not shown) consistent with the presence of function heat shock proteins. This increased activity was further confirmed as providing a growth promoting effect as described below.

Example 10: Impact of the removal of Hsps from rice recombinant albumin on cell viability.

Methods: The separation scheme described in Example 9 was also used to produce fraction A suitable for cell culture testing (**FIG. 12**). The method involves minimal manipulation of fraction A, as it is flowed through an ATP agarose column and then concentrated by diafiltration and buffered with PBS that is suitable for cell culture. The intent of the method is to not introduce new variables into the experiment such that a loss of viability is seen but due to some other reason or cause beyond the removal of ATP binding proteins. Fraction A was tested against the unadulterated control (starting material) for ability to promote hybridoma cell culture viability. The results of the test are shown in **FIG. 12**.

Results: As shown in **FIG. 12** the Cellastim starting material (cross hatched bars), and Part A (solid bars) were tested at the same concentration and compared to the negative control (striped bars). A statistically significant decrease is observable at all four concentrations tested The result indicates that there was a significant loss in the performance of Cellastim after ATP agarose treatment. The treatment resulted in a 28.0, 21.7, 26.7, and 79.5 % loss as compared to Cellastim before removal of ATP binding proteins. In this experiment, care was taken in the

design and handling of the samples to ensure that any inadvertent losses in performance due to sample handling or the accidental introduction of new contaminants were minimized.

Discussion: The cell culture results (**FIG. 12**) demonstrate that it is possible to reduce the performance of the rice derived recombinant albumin by simply passing it over an ATP binding column. This data, when combined with the results shown in Example 9 demonstrates that the depletion of the hsps from albumin by the ATP agarose column directly reduces the cell growth promoting properties of the albumin. This result therefore demonstrates that the superior properties of the albumin arise, at least in part, from the contaminating heat shock proteins in the albumin.

Example 11: Effect of rice derived recombinant human serum albumin in combination with lactoferrin on the proliferation of CHO K1 in serum free culture.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components when added in combination with these components, a series of additional experiments were designed to evaluate and identify optimum combinations of such components.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 μM methotrexate, 50 μg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 μg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5×10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or rLF or various mixtures of the two additives. Control cultures without added rHSA or rLF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies). Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. All cultures were grown at 37°C, 6% CO_2 in a humidified environment. The Observed Effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Table E9 shows the number of viable cells after 6 days of culture Cells in media without supplementation proliferated to 3.3 x 10^5 viable cells/ml. Addition of plant derived recombinant human serum albumin (rHSA) or recombinant lactoferrin (rLF), or a combination of the two increased the proliferation of cells as indicated below. Column two shows the Observed Effect of the respective additive(s) above baseline control culture. Column 3 shows the Predicted Effect using a model where the predictive additive effect equals the sum of additive1 and additive2.

TABLE E9				
	Viable cells(e^5)/ml	**Observed Effect**	**Predicted Effect**	**Ratio of Lactoferrin to albumin**
No Additives (Baseline)	3.3	0		
rHSA 0.5 g/L	7.3	4.0		
rHSA 1 g/L	7.9	4.6		
rLF 0.1 g/L	7.5	4.2		
rLF 0.8 g/L	16.0	12.7		
rLF 0.1 g/L +rHSA 0.5 g/L	18.9	15.6	8.2	1 : 5
rLF 0.8 g/L +rHSA 0.5 g/L	29.0	25.7	16.7	1 : 0.625
rLF 0.1g/L +rHSA 1g/L	23.6	20.3	8.8	1 : 10
rLF 0.8g/L +rHSA 1g/L	35.4	**32.1**	17.3	. 1.25

Discussion: Lactoferrin is an essential factor for serum free cell culture media, where it acts to transport iron, additionally Lactoferrin has more recently been identified as a growth factor and inhibitor of apoptosis in some cell types (Huang et al., In vitro Cell. Dev. Biol. Anim. (2008) 44(10) 464-71; Wong et al., Rheumatology 48(1) 39-44; Bi et al., Arch. Immunol. Ther. Exp. (1997)45(4) 315-20,Hashizume et al., Biochem. Biophys. Acta. (1983) 763(4) 377-82). In **Table E9**, the data shows that the addition of 0.1 g/L and 0.8 g/L recombinant lactoferrin resulted in a significant enhancement of cell growth compared to the baseline rate of cell growth consistent with lactoferrins growth stimulating properties.

The growth stimulating effect of lactoferrin was further enhanced by the addition of plant derived recombinant human serum albumin. Unexpectedly the combination of 0.1 g/L lactoferrin and 0.5 g/L plant derived recombinant human serum albumin (1 to five ratio) resulted in a stimulation in cell growth that was approximately 4 fold higher than either albumin alone or lactoferrin alone

tested at the same concentrations. While the combination of 0.1 g/L lactoferrin and 1.0 g/L human serum albumin (1 to 10 ratio) resulted in a stimulation in cell growth that was approximately 4.4 to almost 5 fold higher than either albumin or lactoferrin alone at the same concentrations.

Synergism was also observed when plant derived recombinant human serum albumin was added to the lactoferrin at a concentration of 0.8 g/L. Even at this significantly higher concentration, the addition of plant derived recombinant human serum albumin further increased cell growth by 2 to 2.5 fold

Albumin and lactoferrin are distinct protein families with little overlapping structural or functional similarity. Thus the fact the synergism was observed with these proteins is unexpected based on their dissimilar structure & function. Synergism provides the opportunity to develop improved supplements that provide for supplements with enhanced cell culturing properties (i.e. improved cell growth and viability) by using optimal ratios of lactoferrin and albumin.

Example 12: Effect of rice derived recombinant albumin in combination with lactoferrin on the productivity of CHO K1 cells grown in serum free culture

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) in 14 ml medium in 125 ml flasks. CHO K1 line DP12 expresses a humanized monoclonal antibody as a secreted product. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and plant derived recombinant lactoferrin (rLF) (Examples 1 & 4) alone or in combination were examined. Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1.5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of plant derived recombinant human serum albumin or LF or various mixtures of the two additives. Control cultures without added HSA or LF were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture. Antibody productivity was determined at the end of the batch culture when cell viability had declined to <10% (typically day 13 or 14) by quantitative ELISA (Bethyl Laboratories). All cultures were grown at 37°C, 6% CO_2, in a humidified environment. The

Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

TABLE E10				
	Product Conc. (µg/ml)	**Observed Effect**	**Predicted Effect**	**Ratio of HSA to Lactoferrin**
No Additives (Baseline)	47.1	0		
rHSA 0.5g/L	114.6	67.5		
rHSA 1g/L	136.1	89.0		
rLF 0.1g/L	91.9	44.8		
rLF 0.8 g/L	138.5	91.4		
rLF 0.1g/L + rHSA 0.5g/L	183.7	136.6	112.3	1 : 5
rLF 0.8g/L + rHSA 0.5g/L	242.1	195.0	158.9	1 : 0.625
rLF 0.1g/L + rHSA 1g/L	219.3	172.2	133 8	1 : 10
rLF 0.8g/L + rHSA 1g/L	260.2	213.1	180 4	1. 1 25

Table E10 shows the effect of plant derived recombinant human serum albumin and recombinant lactoferrin produced in monocot cells, and various combinations of the two proteins on the productivity of CHO K1 cells. Table E10 shows that the concentration of antibody product produced by the cells after 13 days of culture in the baseline medium (with no additives) produced 47.1 µg/ml of antibody product. Medium supplemented with recombinant albumin or recombinant lactoferrin alone or a combination of plant derived recombinant human serum albumin and lactoferrin produced significantly higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the predictive additive effect of the combination of two additives using a model where the predictive additive equals the sum of each additive alone at the same concentration.

Discussion: Table E10 demonstrates that improvements in cell growth and viability directly translated into improved antibody expression and cell productivity. As was the case for cell viability and cell growth, maximal effects of the protein components was observed when these were added to the culture together.

Table E11 shows an additional data set showing the effect of the additives at an expanded range of concentrations, using the same conditions as in **Example12.** Medium supplemented with recombinant albumin alone, or lactoferrin alone, or a combination of both proteins, produced higher levels of antibody than the control medium with no additives. Column 2 shows the Observed Effect of the additive(s) over the baseline control medium. Column 3 shows the Predicted Effect of the combination of two additives using a model where the Predicted Effect equals the sum of the effects of each additive when added alone at the same concentration. The data indicates that the actual effect of the combination is greater than the Predicted Effect over all of the ratios of the two proteins tested.

TABLE E11

	Product Conc. (ug/ml)	Observed Effect	Predicted Effect	% Improvement over predicted activity	Ratio of Lactoferrin to HSA
No additives (Baseline)	56.9	0.0			
Single additives					
HSA 0.5g/L	99.1	42 2			
HSA 1 g/L	126.3	69.4			
HSA 2 g/L	186.0	129.1			
HSA 3 g/L	212.9	156.0			
LF 0.1g/L	123.1	66.2			
LF 0.5g/L	173.5	116.6			
LF 0.8g/L	182.0	125.1			
LF 1.5g/L	196.3	139.4			
Combinations					
LF 0.1g/L+ HSA 0.5g/L	212.4	155.5	108.5	143%	1 : 5
LF 0.5g/L+ HSA 0.5g/L	314.9	258.0	158.9	163%	1: 1
LF 0.8g/L+ HSA 0.5g/L	302 8	245.9	167.4	147%	1: 0 625
LF 1.5g/L+ HSA 0.5g/L	336 8	279.9	181.7	154%	1: 0 33

LF 0.1g/L+ HSA 1.0g/L	239 7	182.8	135.7	133	1: 10
LF 0.5g/L+ HSA 1.0g/L	339 4	282.5	186.1	152	1: 2
LF 0.8g/L+ HSA 1 0g/L	357.7	300.8	194.6	155	1: 1.2
LF 1 5g/L+ HSA 1 0g/L	343.7	286.8	208.9	137	1: 0.66
LF 0.1g/L+ HSA 2.0g/L	261.3	204.4	195.4	105	1: 20
LF 0.5g/L+ HSA 2.0g/L	377 2	320.3	245.8	131	1: 4
LF 0.8g/L+ HSA 2.0g/L	414 8	357.9	254.3	141	1: 2.4
LF 1.5g/L+ HSA 2.0g/L	358.0	301.1	268.6	112	1: 1.33

Discussion: Ratios of lactoferrin to albumin as low as 1 to 0.333 to as high as 1: 20 produced synergistic stimulations of cell growth. Optimal ratios of lactoferrin to albumin were found at 1 to 0.333 wt/wt (at an albumin concentration of 0.5 g/L), at 1 to 1.2 wt/wt (at an albumin concentration of 1.0 g/L); and at 1: 2.4 wt/wt (at an albumin concentration of 2.0 g/L).

Thus the stimulation of cell productivity occurs over a broad range of ratios of recombinant albumin and lactoferrin. Optimal ratios of lactoferrin and recombinant albumin vary to a certain extend on the absolute concentration of both protein components. Preferred ratios of Lactoferrin to Albumin range from about 1 (lactoferrin) to 0.33(albumin) to about 1 (lactoferrin) to 3 (albumin) wt/wt.

Example 13: Evaluation of the effect of combinations of albumin and transferrin in the presence of other cell culture components.

To determine the effect of other cell culture components on the synergistic effect of lactoferrin and albumin on cell growth and viability, a series of additional experiments were carried out with other cell culture components which have been previously shown to be essential for cell growth in serum free conditions.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4×10^5 viable cells/mL. In these experiments transferrin (TF) was used at an initial concentration of 5.5 µg/mL; Plant derived recombinant

Human Serum Albumin (HSA) was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite (SE) and Ethanolamine were used at concentrations of 1:500,000 v/v and 6 7 µg/mL respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the supplements listed above, as described in **Table E12** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C. 6% CO_2, in a humidified environment.

TABLE E12

	Viable cells/ ml	Std	Observed Effect	Predicted Effect	Synergistic	Ratio of transferrin to albumin
DMEM/F12 alone	0.01	0.01				
10% FBS	11.40	0 71	11.39			
TF 5.5 µg/L	0.02	0.00	0.01			
SE 6.7 µg/L	0.22	0.13	0.21			
HSA 0.1 g/L	2.31	0.06	2.30			
HSA 0.5 g/L	4.67	0.18	4.66			
HSA 1 g/L	4.55	0.46	4.54			
doubles						
TF+SE	0.40	0.04	0.38	0.21	yes	
TF+ HSA 0.1 g/L	3.00	0.02	2.98	2.30	yes	1:18
TF+ HSA 0.5 g/l	5.42	0.20	5.41	4.67	yes	1:90
TF+ HSA 1 g/L	5.71	0.14	5.69	4.55	yes	1:181
doubles						
SE+ HSA 0.1 g/l	2.76	0 06	2.75	2.50	Yes/no	
SE+ HSA 0.5 g/L	4.15	0.16	4.14	4.87	no	

SE+ HSA 1 g/L	3.75	0.22	3.74	4.75	no	
Triples						
TF+SE+ HSA 0.1 g/L	7.40	0.00	7.39	2 51	yes	*1:18*
TF+SE+ HSA 0.5 g/L	9.48	0.31	9.46	4.87	yes	*1:90*
TF+SE+ HSA 1 g/L	9.71	0.11	9.70	4.75	yes	*1 181*

Discussion: Recombinant human serum albumin in combination with recombinant transferrin showed synergistic effects when added to culture media together. This effect was further augmented in the presence of selenium. However the addition of selenium did not act synergistically with recombinant plant derived human serum albumin alone. Maximal synergistic effects of transferrin and plant derived recombinant human serum albumin occurred in the presence of selenium and at ratios of transferrin to plant derived recombinant human serum albumin of about 1 (transferrin) to 100 (albumin) wt/wt. and of about 1 (transferrin) to 200 (albumin).

Selenium is a well known essential trace element for cell growth and development, and its positive role in biological systems includes detoxification of free radicals by activating glutathione peroxidase. Additionally selenium can act as a highly effective iron carrier in serum free media, which helps to explain its synergistic impact on transferrin stimulated cell growth. (Zhang et al., Biotechnol. Bioeng. (2006) 95(6) 1188-97)

Example 14: Evaluation of the effect of combinations of albumin, transferrin and insulin.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin, plant derived human recombinant serum albumin, sodium selenite and ethanolamine alone and in combination with insulin was examined on cells which were seeded into DMEM/F12 at an initial cell density of 0.4x 10^5 viable cells/mL. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 0.1 g/L, 0.5 g/L, and 1 g/L & Sodium Selenite and Ethanolamine were used at concentrations of 6.7 µg/mL and 1:500,000 v/v and respectively. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E13** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment, and shaken with 2 cm rotational orbit at 120 RPM.

TABLE E13

	Viable cells/ml	Std	Observed Effect	Predicted Effect	Synergistic
D/F12 alone	0.01	0.01			
10% FBS	11.65	0.21	11.64		
Factors Alone					
Insulin (IN) 10 µg/L	0.04	0.02	0.03		
Transferrin (TF) 5.5 µg/L	0.02	0.00	0.01		
Sodium Selenite (SE) 6.7 µg/L	0.22	0.13	0.21		
HSA 0.1 g/L	2.31	0.06	2.30		
HSA 0.5 g/L	4.67	0.18	4.66		
HSA 1 g/L	4.55	0.46	4.54		
Doubles					
IN+TF	0.03	0.02	0.02	0.06	
IN+SE	1.06	0.15	1.05	0.26	Yes
IN+ HSA 0.1 g/L	2.54	0.08	2.53	2.35	No
IN+ HSA 0.5 g/L	3.96	0.34	3.95	4.71	No
IN+ HSA 1 g/L	4.61	0.16	4.60	4.59	No
Triples					
IN+TF+SE	2.13	0.49	2.12	0.28	Yes
IN+TF+ HSA 0.1 g/L	3.29	0.03	3.28	2.37	Yes
IN+TF+ HSA 0.5 g/L	5.84	0.49	5.83	4.73	Yes
IN+TF+ HSA 1 g/L	6.84	0.34	6.83	4.61	Yes

Triples					
IN+SE+ HSA 0.1 g/L	2.21	0.17	2.19	2.37	No
IN+SE+ HSA 0 5 g/L	3.41	0.21	3.39	4.73	No
IN+SE+ HSA 1 g/L	3 62	0.23	3.60	4.61	No
Quaternaries					
IN+TF+SE+ HSA 0 1 g/L	7.91	0.46	7 90	2 59	Yes
IN+TF+SE+ HSA 0 5 g/L	9.14	0.63	9 13	4 95	Yes
IN+TF+SE+ HSA 1 g/L	10.07	0.67	10.05	4.83	Yes

Discussion: In these studies the addition of insulin and plant derived recombinant human serum albumin did not produce a synergistic effect on cell growth. By contrast the addition of plant derived recombinant human serum albumin and transferrin and insulin did produce a synergistic effect on cell growth.

Example 15: Evaluation of the effect of combinations of albumin, transferrin and insulin in the presence of Selenium.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin,(HSA) insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 6 7 µg/m L Sodium Selenite at 0.4x 10^5 viable cells/mL In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL, transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL Ethanolamine were used at a concentration of 1·500,000 v/v The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E14** below. Control cultures without added supplements were used as the baseline All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E14						
Condition	Viable cell count 10e5	Std	Observed Effect	Predicted Effect	% Improvement over predicted activity	Synergistic
Base medium of D/F12+ Selenium	0.08	0.04	0.00			
10% FBS	13.20	0 28	13.12			
Insulin	0.30	0.09	0.22			
Factors Alone						
TF	0.27	0.04	0.19			
HSA 0.1 g/L	2.23	0.11	2.15			
HSA 0.5 g/L	6.21	0.20	6 13			
HSA 1 g/L	8.00	0 05	7 91			
doubles						
IN+TF	0.48	0.02	0.40	0.41		No
IN+ HSA 0.1 g/L	1.81	0.02	1.72	2.37		No
IN+ HSA 0.5 g/L	6.00	0.09	5.91	6.35		No
IN+ HSA 1 g/L	7.43	0 19	7.35	8.13		No
doubles						
TF+ HSA 0 1 g/L	4.29	0.04	4.20	2.34	179	Yes
TF+ HSA 0 5 g/L	12.35	0.21	12.27	6.32	194	Yes
TF+ HSA 1 g/L	15.48	0.71	15.39	8.1	190	Yes
Triples						
IN+TF+ HSA 0.1 g/L	4.18	0.21	4.09	2.56	159	Yes
IN+TF+ HSA 0.5 g/L	13.60	0.00	13.52	6.54	206	Yes
IN+TF+ HSA 1 g/L	15.38	0 22	15.29	8.32	184	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin.

Example 16: Evaluation of the effect of combinations of albumin and transferrin in the presence of Ethanolamine.

Methods: Hybridoma cells actively growing in DMEM/F12 medium with 10% FBS were washed three times in DMEM/F12 without FBS to remove FBS. The growth and productivity enhancing effect of plant derived transferrin (TF), plant derived recombinant human serum albumin (HSA), insulin, sodium selenite and ethanolamine alone and in combination was examined in base medium containing 1:500,000 Ethanolamine v/v at 0.4x 10^5 viable cells/ml. In these experiments recombinant human Insulin (IN) (Millipore) was used at a concentration of 10 µg/mL transferrin (TF) was used at an initial concentration of 5.5 µg/mL; plant derived recombinant Human Serum Albumin was used at initial concentrations of 100 µg/mL, 500 µg/mL, and 1000 µg/mL & Sodium Selenite was used at a concentration of 6.7 µg/mL. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components.

Shake cultures were further supplemented with various concentrations of the factors listed above, as described in **Table E15** below. Control cultures without added supplements were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined after 3 days via a Guava PCA cell counter (Guava Technologies). All cultures were grown at 37°C, 6% CO_2, in a humidified environment.

TABLE E15				
Condition	Observed Effect (Viable cells/ml)	Std	Predicted Effect (Viable cells/ml)	Synergistic
Base Medium of D/F12+ Ethanolamine	0.51	0.16		
10% FBS	14.45	0.64		
IN	1.41	0.24		
Factors Alone				
TF	2.08	0.15		
HSA 0.1 g/L	2.16	0.07		
HSA 0.5 g/L	5.66	0.35		
HSA 1 g/L	7.08	0.21		
doubles				
IN+TF	3.12	0.11	3.48	No
IN+ HSA 0.1 g/L	1.87	0.00	3.57	No
IN+ HSA 0.5 g/L	4.48	0.25	7.07	No
IN+ HSA 1 g/L	5.14	0.19	8.49	No
doubles				
TF+ HSA 0.1 g/L	6.72	0.35	4.24	Yes
TF+ HSA 0.5 g/L	12.30	0.26	7.74	Yes
TF+ HSA 1 g/L	16.20	0.65	9.16	Yes
Triples				
IN+TF+ HSA 0.1 g/L	7.46	0.28	5.64	Yes
IN+TF+ HSA 0.5 g/L	13.30	0.36	9.15	Yes
IN+TF+ HSA 1 g/L	16.60	0.48	10.57	Yes

Discussion: Again, as shown in the last experiment, combinations of plant derived recombinant human serum albumin and insulin did not produce a synergistic stimulation of cell growth when these two components were added to the cell culture media together. By comparison synergism was observed between mixtures of plant derived recombinant human serum albumin and transferrin, over all concentration ranges tested. Synergism was also observed with plant derived recombinant human serum albumin and transferrin in the presence of insulin under these conditions.

Surprisingly the combination of Insulin, transferrin and plant derived recombinant human serum albumin produced superior cell growth compared to 10 % serum, demonstrating the superior properties of these combinations of proteins.

Example 17: Comparison of rice recombinantly produced albumin and plasma derived albumin.

To directly compare the effect of rice derived recombinant human serum albumin (rHSA) to plasma derived serum albumin (pHSA), samples of both were directly compared at two ratios when mixed with rice derived recombinant transferrin for their ability to stimulate cell growth.

Methods: Hybridoma cells were grown in DMEM/F12 (Gibco) in 10% FBS. Cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in either 1)DMEM/F12 base medium, 2) DMEM/F12+sodium selenate base medium or 3) DMEM/F12 base medium at $0.75x10^5$ viable cells/ml. Each base medium was further supplemented with either recombinant transferrin (TF), rice derived recombinant human serum albumin (rHSA), plasma derived human serum albumin (pHSA) or combinations of both transferrin and either form of albumin. After 3 days of incubation at 37° C, 5% CO_2, the concentration of viable cells was determined by a Guava PCA cell counter as instructed by the manufacturer. The Observed effect was calculated as the net viable cell concentration (observed-base medium). The Predictive Effect was calculated as the sum of the effect of the individual components. The Effect Factor was calculated as the observed effect divided by the expected effect. Effect factor of 1 is indicative of expected effect (additive). Effect factor >1 is indicative of more than additive or synergistic effect.

In these experiments transferrin (TF) was used at an initial concentrations of 1 and 3 mg/L; albumins were used at concentrations of 5 g/L, Sodium Selenite was used at a concentration of

6.7 µg/L & Ethanolamine was used at 1:500,000 v/v.

Table E16					
Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual / expected
Base Medium	0.11	0.07			
Components alone					
rTF 1 mg/L	0.21	0.01	0.10		
rTF 3 mg/L	0.28	0.09	0.18		
pHSA 5 g/L (Seracare)	11.30	0.28	11.19		
rHSA 5 g/L (Cellastim)	5.11	0.02	5.00		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	12.85	0.49	12.74	11.51	1.1
rTF3 mg/L + pHSA 5 g/L	13.20	0.42	13.09	16.41	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	18.80	0.71	18.69	5.31	3.5
rTF3 mg/L + rHSA 5 g/L	18.85	0.92	18.74	5.39	3.5

Base Medium:DMEM/F12	Viable cells/ml 10e5	Std	Observed Effect	Predicted Effect	Effect Factor (actual/expected
Base Medium	0.04	0.02			
Components alone					
rTF 1 mg/L	0.14	0.01	0.10		
rTF 3 mg/L	0.14	0.05	0.10		
pHSA 5 g/L	6.08	0.13	6.04		
rHSA 5 g/L	1.76	0.17	1.72		

pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	7.46	0.49	7.42	6.22	1.2
rTF3 mg/L + pHSA 5 g/L	7.84	0.05	7.79	7.84	1.0
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	8.55	0.79	8.51	1.90	4.5
rTF3 mg/L + rHSA 5 g/L	8.37	0.23	8.33	1.90	4.4

Base Medium:DMEM/F12	**Viable cells/ml 10e5**	**Std**	**Observed Effect**	**Predicted Effect**	**Effect Factor (actual/ expected**
Base Medium	1.01	0.02			
Components alone					
rTF 1 mg/L	3.45	0.27	2.44		
rTF 3 mg/L	3.47	0.27	2.46		
pHSA 5 g/L	13.15	0.49	12.14		
rHSA 5 g/L	3.46	0.13	2.45		
pHSA combinations					
rTF 1 mg/L + pHSA 5 g/L	14.83	0.49	13.82	16.60	0.8
rTF3 mg/L + pHSA 5 g/L	14.57	0.12	13.56	16.61	0.8
rHSA combinations					
rTF1 mg/L + rHSA 5 g/L	17.87	1.46	16.86	6.91	2.4
rTF3 mg/L + rHSA 5 g/L	20.20	1.13	19.19	6.93	2.8

Discussion: Table E14 shows the results of three independent experiments that confirm that the combinations of TF with rice derived recombinant human serum albumin produced Observed Effect values much greater than the Predicted Effect. By comparison, the combination of ***plasma***-derived HSA and transferrin produced values that were similar to, or less than, the expected effect. Thus the ability of albumin to synergistically enhance the activity of transferrin

and lactoferrin appears to be dependent on the source of albumin, rather than being an inherent characteristic of the albumin itself Specifically the present experiments establish that the synergistic properties of human serum albumin with transferrin related proteins such as transferrin and lactoferrin is an unexpected and surprising property of rice derived recombinant human serum albumin. Significantly the combination of transferrin and plant derived recombinant albumin was more effective in stimulating cell growth than the combination of plasma derived albumin and transferrin, even though plasma derived albumin was more effective at 5 g/L alone than the same concentration of rice derived human serum albumin.

Example 18: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with either plasma derived or recombinant transferrin.

To directly compare the effect of rice derived recombinant human transferrin (rTF) to plasma derived transferrin (pTF), samples of both were directly compared when mixed with rice derived recombinant human serum albumin for their ability to stimulate cell growth.

Methods: A base medium (BM) was composed that consisted of DMEM/F12+ with Sodium Selenite 0.0067 mg/L, Ethanolamine 1:500,000 v/v. Base medium included human plasma transferrin, pTF at a concentration of 5.5 mg/L in combination with either plasma HSA (pHSA, cell culture grade Seracare) or rice derived recombinant HSA (rHSA). Hybridoma cells in log-phase growth were harvested, washed three times in DMEM/F12 to remove FBS, and seeded in at $0.80x10^5$ viable cells/ml and incubated at 37° C, 6% CO_2, The concentration of viable cells was determined daily by a Guava PCA cell counter as instructed by the manufacturer. The concentration of antibody produced by the cells and secreted into the medium was determined on day 6 by quantitative ELISA (Bethyl laboratories) Error bars indicate standard deviation The production of antibody with the combination of pTF +rHSA was similar to that of DMEM/F12+10% FBS.

Discussion: FIG. 13 shows that rice derived recombinant human serum albumin was able to act synergistically with plasma derived transferrin to significantly enhance the growth and productivity of the cells. By comparison, plasma derived serum albumin was unable to exert a similar stimulatory effect. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective with transferrin related proteins that were either plasma derived, or recombinant.

Example 19: Evaluation of ability of rice derived recombinant albumin to stimulate cell growth in combination with insulin related growth factor-1.

To investigate whether the unique attributes of plant derived human serum albumin (Cellastim) could positively impact the growth promoting activities of other cell culture components such as the growth factor IGF-1, additional experiments were performed to assess this possibility.

Methods: CHO K1 line DP12 clone 1934 (ATCC# CRL-12445) was maintained in SAFC 325 PF protein free CHO medium supplemented with 0.5% FBS, 1 µM methotrexate, 50 µg/mL penicillin/streptomycin, 1mM Glutamax (Invitrogen) as described previously. The growth and productivity enhancing effect of plant derived recombinant human serum albumin (rHSA) (Examples 3 & 5) and recombinant insulin related growth factor 1 (IGF1) (Ajinomoto) alone or in combination were examined Cells were washed twice in 325 PF CHO medium containing 200 nM methotrexate, 50 µg/mL penicillin/streptomycin and seeded in the same medium into 4 ml shake-batch cultures at 1 5 x 10^5 cells/ml. 4 ml shake cultures were further supplemented with various concentrations of rHSA or IGF-1 or various mixtures of the two additives. Control cultures without added rHSA or IGF-1 were used as the baseline. All culture conditions were performed as duplicates. The concentration of viable cells was determined daily for the 14-day batch period via a Guava PCA cell counter (Guava Technologies) Log-phase growth was judged by the relative proliferation of the cells after 6 days of culture All cultures were grown at 37°C, 6% CO_2, in a humidified environment. Table E17 shows the results of the experiment. Here the Observed Effect was calculated as the net viable cell concentration (observed-base medium), and the Predictive Effect was calculated as the sum of the effect of the individual components.

Table E17

	viable cells/ml 10e5	Observed Effect (Cell count-baseline)	Predicted Effect
No Additive(baseline)	3 3	0.0	
HSA 0.5 g/L	7.3	4 0	
HSA 1g/L	7.9	4.6	
IGF-1 30 nM	12.0	8.7	

HSA 0.5 g/L+ IGF-1 30nM	23.5	15.6	12.7
HSA 1 g/L+ IGF-1 30nM	25.6	13.6	13.3

Results and Discussion: **Table E17** shows that rice derived recombinant human serum albumin was able to act synergistically with recombinant IGF-1 to significantly enhance the growth and productivity of the cells. By comparison, recombinant albumin did not act synergistically with insulin (Table E15) suggesting that synergism is not a general effect across all cell culture components. Specifically the present experiments establish that the synergistic properties of rice derived human serum albumin are also effective in combination with IGF-1.

It will, of course, be appreciated that the above description has been given by way of example only and that modifications in detail may be made within the scope of the present invention.

The invention is capable of considerable modification, alteration, and equivalents in form and function, as will occur to those ordinarily skilled in the pertinent arts having the benefit of this disclosure.

While the present invention has been described for what are presently considered the preferred embodiments, the invention is not so limited. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the detailed description provided above.

ABSTRACT OF THE DISCLOSURE

The present invention relates to cell culture media containing combinations of proteins, as well as methods of making the cell culture media, and methods of using the cell culture media to improve growth characteristics of cultured cells.

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Applicant : Pettit
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- [X] Specification filed 07/09/2018, page(s) 101-103 (illegible tables).
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[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(c) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

Electronic Acknowledgement Receipt

EFS ID:	38749797
Application Number:	15188478
International Application Number:	
Confirmation Number:	8304
Title of Invention:	CELL CULTURE MEDIA CONTAINING COMBINATIONS OF PROTEINS
First Named Inventor/Applicant Name:	Steven Clyde Pettit
Customer Number:	23589
Filer:	Crissa A. Cook/Brandy Goodman
Filer Authorized By:	Crissa A. Cook
Attorney Docket Number:	50091-CNT1
Receipt Date:	03-MAR-2020
Filing Date:	21-JUN-2016
Time Stamp:	09:51:15
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		PTO_Amendment_03Mar20_50091-CNT1_1460670.PDF	128016	yes	3

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Amendment after Notice of Allowance (Rule 312)		1	1	
	Specification		2	2	
	Applicant Arguments/Remarks Made in an Amendment		3	3	
Warnings:					
Information:					
2		PTO_Substitute_Spec_Clean_03Mar20_50091-CNT_1460366.PDF	997648	yes	126
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	125	
	Abstract		126	126	
Warnings:					
Information:					
3	Miscellaneous Incoming Letter	PTO_Notice_to_File_Corrected_App_Papers_28Feb2020_50091-US-CNT1_1459462.PDF	98842	no	3
Warnings:					
Information:					
		Total Files Size (in bytes):	1224506		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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UNITED STATES DEPARTMENT OF COMMERCE
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/188,478	06/21/2016	Steven Clyde Pettit	50091-CNT1	8304

23589 7590 03/17/2020
Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

EXAMINER	
HOLLAND, PAUL J	

ART UNIT	PAPER NUMBER
1656	

NOTIFICATION DATE	DELIVERY MODE
03/17/2020	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

uspatents@hoveywilliams.com

Response to Rule 312 Communication	Application No. 15/188,478	Applicant(s)
	Examiner	Art Unit

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☒ The amendment filed on *03 March 2020* under 37 CFR 1.312 has been considered, and has been:

a) ☒ entered.

b) ☐ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

Publishing Division

Charles Bowen

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)
Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	15188478
Filing Date	2016-06-21
First Named Inventor	Steven Clyde Pettit
Art Unit	1656
Examiner Name	Paul J. Holland
Attorney Docket Number	50091-CNT1

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6733746		2004-05-11	Daley	
	2	4075197		1978-02-21	Schuck	
	3	4086222		1978-04-25	Lindquist	
	4	4093612		1978-02-27	Travis	
	5	4097473		1978-06-27	Lewis	
	6	4136094		1979-01-23	Condie	
	7	4228154		1980-10-14	Fisher	
	8	5250662		10-1993 ~~1992-02-27~~	Chang	

(Change(s) applied to document.
A.C.
2/20/2020

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.J.H/

EFS Web 2.1.17

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Alexandria, Virginia 22313-1450
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APPLICATION NO	ISSUE DATE	PATENT NO	ATTORNEY DOCKET NO.	CONFIRMATION NO
15/188,478	04/14/2020	10618951	50091-CNT1	8304

23589 7590 03/25/2020

Hovey Williams LLP
10801 Mastin Blvd., Suite 1000
Overland Park, KS 66210

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Ventria BioScience Inc., Fort Collins, CO
Steven Clyde Pettit, Fort Collins, CO;
Mary Ann Michelle Fernandez Santos, Elk Grove, CA;
Ning Huang, Davis, CA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

IR103 (Rev. 10/09)

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court for the District of Kansas on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
5:21-cv-04008	2/5/2021	for the District of Kansas

PLAINTIFF	DEFENDANT
Ventria Bioscience Inc.	Wuhan Healthgen Biotechnology Corp.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	10,618,951	4/14/2020	Ventria Bioscience Inc.
2	8,609,416	12/17/2013	Ventria Bioscience Inc.
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED	INCLUDED BY ☐ Amendment ☐ Answer ☐ Cross Bill ☐ Other Pleading	
PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1		
2		
3		
4		
5		

In the above—entitled case, the following decision has been rendered or judgement issued

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy