

Methods for Improving Viability and Productivity in Cell  
Culture

Field of the Invention

5           The present invention relates to methods of increasing viability and production of secreted proteins in fed batch eukaryotic cell culture.

Background of the Invention

10           Mammalian cell culture is the system of choice for many recombinant protein production processes due to its ability to produce proteins with proper post-translational modifications. With increasing manufacturing demand, a strong motivation exists to improve process efficiency by  
15           increasing product yield. Attaining the grams per liter production levels of biotherapeutics in commercial production processes relies upon the optimization of both mammalian cell culture and engineering methods.

          Inherent in current high density, protein-free mammalian  
20           cell cultures is the problem of cell death of which apoptosis can account for up to 80% in a typical fed-batch bioreactor, induced in response to stressors such as nutrient and growth factor deprivation, oxygen depletion, toxin accumulation, and shear stress (Goswami et al., Biotechnol. Bioeng. 62:632-640  
25           (1999)). Apoptosis limits the maximum viable cell density, accelerates the onset of the death phase and potentially decreases heterologous protein yield (Chiang and Sisk, Biotechnol. Bioeng. 91:779-792 (2005); Figueroa et al., Biotechnol. Bioeng. 73:211-222 (2001), Metab. Eng. 5:230-245  
30           (2003), Biotechnol Bioeng. 85:589-600 (2004); Mercille and Massie, Biotechnol. Bioeng. 44:1140-1154 (1994)).

          Apoptosis is a result of a complex network of signaling pathways initiating from both inside and outside the cell, culminating in the activation of caspases that execute the  
35           final stages of cell death. Various methods of apoptosis prevention have been used to maintain cell viability during extended production runs in mammalian cell culture (Arden and

Betenbaugh, Trends Biotechnol. 22:174-180 (2004); Vives et al., Metab. Eng. 5:124-132 (2003)). Altering the extracellular environment through media supplementation of growth factors, hydrolysates, and limiting nutrients has led to increased protein production and decreased apoptosis (Burteau et al., In Vitro Cell Dev. Biol. Anim. 39:291-296 (2003); Zhang and Robinson, Cytotechnology 48: 59-74 (2005)). Other researchers have turned to chemical and genetic strategies to inhibit the apoptotic signaling cascade from within the cell (Sauerwald et al., Biotechnol. Bioeng. 77:704-716 (2002), Biotechnol. Bioeng. 81:329-340 (2003)). Researchers have found that over-expression of genes found upregulated in cancer cells can prolong viability in cells grown in bioreactors by preventing apoptosis upstream of caspase activation (Goswami et al., supra; Mastrangelo et al., Trends Biotechnol. 16:88-95 (1998); Meents et al., Biotechnol. Bioeng. 80:706-716 (2002); Tey et al., J Biotechnol. 79:147-159 (2000) and Biotechnol. Bioeng. 68:31-43 (2000)).

The anti-apoptotic genes that function in the mitochondrial apoptotic pathway can be divided into three groups, namely 1) those that act early in the pathway, e.g., members of the Bcl-2 family of proteins; 2) those that act mid-pathway to disrupt or inhibit the apoptosome complex, e.g., Aven and 3) those that act late in the pathway, e.g., caspase inhibitors, XIAP. The functionality of the majority of these genes have been studied by over-expressing them in mammalian expression systems, and in some cases the effect of combined over-expression of two or more genes, each derived from a different part of the pathway has been determined. Examples include 1) the additive effect of Bcl-XL and a deletion mutant of XIAP (XIAPΔ) in CHO cells (Figuerola et al., Metab. Eng. 5:230-245 (2003)); 2) E1B-19K and Aven in BHK cells (Nivitchanyong et al., Biotechnol. Bioeng. 98:825-841 (2007)) and 3) Bcl-XL, Aven and XIAPΔ (Sauerwald et al., Biotechnol. Bioeng. 81:329-340 (2003)).

One of the major activators of the apoptosis cascade is the protein p53. One mechanism by which p53 activates

apoptosis is through up-regulation of a subset of pro-apoptosis proteins including BNIP3 (Yasuda et al., J. Biol. Chem. 273:12415-21 (1998)). Therefore, up-regulation of p53 may be one of the principal factors triggering apoptosis.

5 p53 can be degraded in cells through ubiquitin mediated degradation pathway via MDM2 (murine double minute-2 gene) (Bond et al., Current Cancer Drug Target 5:3-8 (2005)). Thus, over-expression of MDM2 has the potential to lower p53 levels and by extension, inhibit apoptosis. Previously, it  
10 was shown that in the presence of stress signals, a CHO cell line over-expressing MDM2 could survive longer in culture compared to wild type CHO in batch culture (Arden et al., Biotechnol. Bioeng. 97:601-614 (2007)).

The various approaches described above to increase  
15 protein productivity have succeeded to varying degrees. Nevertheless, there is a continuous need to develop methods to increase protein production, especially in large-scale commercial production.

## 20 Brief Description of the Drawings

Figure 1. Growth Profiles of cell lines generated from co-transfection of Bcl-2Δ, MDM-2 and XIAPΔ.

Figure 2. Growth Profiles of cell lines generated from co-transfection of Bcl-XL, MDM-2 and XIAPΔ.

25 Figure 3. Growth Profiles of cell lines generated from co-transfection of E1B19K and MDM-2. A) Viable Cell Density; B) Integrated Viable Cell Density; C) Down-regulation of Caspase 3/7

Figure 4. Growth profiles of cell lines transfected with  
30 MDM2<sup>D300A</sup> in shake flask batch culture.

A. Transfected host was 1013A

B. Transfected host was C1835A

Figure 5. Growth profiles of 1013A cell line transfected with DM2<sup>D300A</sup> in shake flask fed-batch culture. A) Viable cell  
35 density; B) % viability.

Figure 6. Antibody titers during fed batch CHO culture.

## Summary of the Invention

One aspect of the invention is a method of increasing cell viability in a fed batch mammalian cell culture, comprising culturing a mammalian cell line over-expressing MDM2<sup>D300A</sup>.

5 Another aspect of the invention is a method of increasing cell viability in a fed batch mammalian cell culture, comprising culturing a mammalian cell line over-expressing MDM2<sup>D300A</sup> and E1B19K.

10 Another aspect of the invention is a method of increasing production of a secreted protein in a Chinese Hamster Ovary (CHO) fed batch cell culture, comprising culturing a CHO cell line over-expressing MDM2<sup>D300A</sup> and one or more genes encoding the secreted protein.

15 Another aspect of the invention is a method of increasing production of a secreted protein in a Chinese Hamster Ovary (CHO) fed batch cell culture, comprising culturing a CHO cell line over-expressing MDM2<sup>D300A</sup> and E1B19K and one or more genes encoding the secreted protein.

20 Another aspect of the invention is a method of increasing production of a secreted protein in a Chinese Hamster Ovary (CHO) fed batch cell culture, comprising culturing a CHO cell line over-expressing MDM2 and E1B19K and one or more genes encoding the secreted protein.

25 Another aspect of the invention is an isolated polynucleotide encoding a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 4.

Another aspect of the invention is an isolated polypeptide comprising a polypeptide having the sequence shown in SEQ ID NO: 4.

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#### Detailed Description of the Invention

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

35 As used herein and in the claims, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a polypeptide" is a reference to one or more

polypeptides and includes equivalents thereof known to those skilled in the art.

The term MDM2 as used herein refers to human MDM2 (MDM2 p53 binding protein homolog) having a polypeptide sequence shown in GenBank accession number NP\_002383 (SEQ ID NOs: 1 and 2).

The term ElB19K as used herein refers to human ElB19K protein, having a polypeptide sequence shown in GenBank accession number NP\_004322 (SEQ ID NOs: 5 and 6).

The term "apoptotic<sup>R</sup> genes" as used herein refers to genes encoding proteins that, when over-expressed in a cell, confer increased resistance to cell death when compared to the untransfected cell. Exemplary apoptotic<sup>R</sup> genes are anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-XL, Bcl-w or ElB19K, caspase inhibitors, for example the IAP family (inhibitors of apoptosis), including XIAP and XIAPΔ, and other proteins involved in cell cycle regulation, for example p27 and MDM2 (Arden et al., BioProcessing J. March/April 23-28 (2004); Sauerwald et al., Bioprocessing J. Summer 2002, 61-68 (2002); Arden et al., Biotechnol. Bioengineer. 97:601-614, (2007)). Cell death can be measured by methods well known in the art, for example by measuring Viable Cell Density (VCD) and percent (%) viability, and by calculating integrated viable cell density (IVCC). Activation of apoptosis can be measured by measuring caspase activity using well known methods.

The term "polypeptide" means a molecule that comprises at least two amino acid residues linked by a peptide bond to form a polypeptide. Small polypeptides of less than 50 amino acids may be referred to as "peptides". Polypeptides may also be referred as "proteins."

The term "polynucleotide" means a molecule comprising a chain of nucleotides covalently linked by a sugar-phosphate backbone or other equivalent covalent chemistry. Double and single-stranded DNAs and RNAs are typical examples of polynucleotides.

The term "complementary sequence" means a second isolated polynucleotide sequence that is antiparallel to a

first isolated polynucleotide sequence and that comprises nucleotides complementary to the nucleotides in the first polynucleotide sequence. Typically, such "complementary sequences" are capable of forming a double-stranded  
5 polynucleotide molecule such as double-stranded DNA or double-stranded RNA when combined under appropriate conditions with the first isolated polynucleotide sequence.

The term "vector" means a polynucleotide capable of being duplicated within a biological system or that can be  
10 moved between such systems. Vector polynucleotides typically contain elements, such as origins of replication, polyadenylation signal or selection markers, that function to facilitate the duplication or maintenance of these polynucleotides in a biological system. Examples of such  
15 biological systems may include a cell, virus, animal, plant, and reconstituted biological systems utilizing biological components capable of duplicating a vector. The polynucleotides comprising a vector may be DNA or RNA molecules or hybrids of these.

20 The term "expression vector" means a vector that can be utilized in a biological system or a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

As used herein, the term "fed batch cell culture" means  
25 a cell culture process which is based on feeding of a growth limiting nutrient substrate to the culture. The fed batch strategy is typically used in bio-industrial processes to reach a high cell density in a bioreactor. Numerous strategies have been devised to improve viability and  
30 ultimately productivity of fed batch cell cultures. In the present invention, an alternative approach is described, whereby a combination of apoptotic<sup>R</sup> genes are over-expressed in a host cell. An unexpectedly high increase in the production of secreted proteins was demonstrated by cells  
35 engineered to over-express both MDM2 and E1B19K in the light of published results describing that expression of MDM-2 alone increased productivity by maximum of 2-fold (Arden et al., Biotechn. Bioengin. 97:601-614, 2007), and expression of

E1B19K, although inhibiting apoptosis and improving cell yields, did not increase production of secreted proteins in a cell (WO2007/124106A2 of Betenbaugh). Further, cell lines generated during the studies described in the Examples below that over-expressed E1B19K only had less than optimal growth properties and low expression levels. Thus, the present invention demonstrated a significant benefit for production of secreted proteins in mammalian cells by co-expressing MDM2 and E1B19K.

10       The present invention also describes a non-naturally occurring mutant MDM2, which is useful in the methods of the invention.

One embodiment of the invention is a method of increasing cell viability in a fed batch mammalian cell culture, comprising culturing a mammalian cell line over-expressing one or more apoptotic<sup>R</sup> genes and evaluating cell viability.

The methods of the invention are useful for increasing viability in mammalian fed batch cell culture, such as Chinese Hamster Ovary (CHO) cell culture, myeloma or hybridoma cell culture. In particular, the methods of the invention are useful for increasing the integrated viable cell count (IVCC) of CHO cell cultures. The cell lines useful in the method of the invention express one or more apoptosis<sup>R</sup> genes. In particular, the genes encoding MDM2 (SEQ ID NOs 1 and 2), MDM2<sup>D300A</sup> (SEQ ID NOs 3 and 4), E1B19K (SEQ ID NOs: 5 and 6), Aven (SEQ ID NOs: 7 and 8), Bcl-LX (SEQ ID NO:s 9 and 10), Bcl-2Δ (SE ID NO: 11 and 12), and XIAPΔ (SEQ ID NOs: 13 and 14) can be used. Expression of the apoptosis<sup>R</sup> genes can be achieved by transfection techniques known to those skilled in the art. The cell lines generated are superior hosts for the development of production cell lines expressing proteins of interest such as peptides, peptide fusions, growth factors, hormones, antibodies, designed ankyrin repeat proteins (DARPs) and other polypeptides useful for therapeutic, diagnostic or research purposes. CHO cell lines useful in the method of the invention include CHO-K1 (Invitrogen, Carlsbad, CA) and CHOK1SV (Lonza Biologics,

Slough, UK). Myeloma lines useful in the method of the invention include NS0 and Sp2/0.

In the present invention, the use of cell lines over-expressing apoptosis<sup>R</sup> genes allows these cell lines to reach  
5 IVCC values about two-fold higher than control cell, increase longevity of fed batch cultures up to 7 days, and improve production of secreted proteins by 2-7 fold. Such improved production is significant and can result in lower production costs for complex biologics and at the same time, generate  
10 product of superior quality due to the absence of cell lysis of the non-viable cells, as lysed cells release proteases that degrade product. Accordingly, these lines are superior hosts for the development of production cell lines expressing a protein or proteins of interest. For example, a CHO cell  
15 line over-expressing MDM2<sup>D360A</sup> reached 2-fold increased IVCC values and survived 7 days longer in culture when compared to control cell line.

Another embodiment of the invention is a method of increasing production of a secreted protein in a CHO fed  
20 batch cell culture, comprising culturing a CHO cell line over-expressing at least one apoptotic<sup>R</sup> genes and one or more genes encoding the secreted protein, and measuring the titer of the secreted protein. Particularly useful cell lines in the methods of the invention are CHO cell lines over-  
25 expressing MDM2 and ElB19K, an a cell line over-expressing MDM2<sup>D360A</sup> alone. Use of these cell lines in the methods of the invention resulted in 5- to 7-fold increased titers of secreted proteins in fed batch culture of up to 21 days.

Over-expression of proteins in a cell can be achieved by  
30 well known methods, either transiently or by stable expression (Davis et al., Basic Methods in Molecular Biology, 2<sup>nd</sup> ed., Appleton & Lange, Norwalk, CT, 1994; Sambrook et al., Molecular Cloning: A Laboratory Manual, 3<sup>rd</sup> ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001).

35 The present invention also provides isolated mutant MDM2 polynucleotides, vectors comprising these polynucleotides, isolated host cells, polypeptides obtainable from expression of these polynucleotides, methods for expressing the

polypeptides of the invention, and methods of using the polynucleotides and polypeptides of the invention. The compositions and methods of the invention can be used for a variety of specific applications. The polynucleotides and  
5 vectors of the invention are useful because they encode mutant MDM2 polypeptides and can be used to express these polypeptides. The mutant MDM2 polypeptides are useful as they can be used to improve cell viability and increase production of secreted proteins in the cell when they are  
10 recombinantly overexpressed or introduced by other means into a host animal or tissue.

One aspect of the invention is an isolated polynucleotide comprising a polynucleotide having the sequence shown in SEQ ID NO: 3 or a complementary sequence  
15 thereof. The polynucleotide sequence shown in SEQ ID NO: 3 encodes a polypeptide comprising the mutant human MDM2<sup>D300A</sup>. In the MDM2<sup>D300A</sup>, a putative caspase cleavage site (AspValProAspCysLysLys) identified in the wild type MDM2 was destroyed to confer MDM2 more resistant to degradation, and  
20 further increase MDM2 levels in the cell during culture. The polynucleotides of the invention may be produced by chemical synthesis such as solid phase polynucleotide synthesis on an automated polynucleotide synthesizer. Alternatively, the polynucleotides of the invention may be produced by other  
25 techniques such as PCR based duplication, vector based duplication, or restriction enzyme based DNA manipulation techniques. Techniques for producing or obtaining polynucleotides of a given known sequence are well known in the art.

30 The polynucleotides of the invention may also comprise at least one non-coding sequence, such as transcribed but not translated sequences, termination signals, ribosome binding sites, mRNA stabilizing sequences, introns and polyadenylation signals. The polynucleotide sequences may  
35 also comprise additional sequences encoding additional amino acids. These additional polynucleotide sequences may, for example, encode a marker or tag sequence such as a hexa-histidine peptide (Gentz et al., Proc. Natl. Acad. Sci. (USA)

86:821-284 (1989) or the HA peptide tag (Wilson et al., Cell 37:767-778 (1984)) which facilitate the purification of fused polypeptides.

Another embodiment of the invention is a vector  
5 comprising an isolated polynucleotide having a sequence shown in SEQ ID NO: 3. The vectors of the invention are useful for maintaining polynucleotides, duplicating polynucleotides, or driving expression of a polypeptide encoded by a vector of the invention in biological systems, including reconstituted  
10 biological systems. Vectors may be chromosomal-, episomal- and virus-derived such as vectors derived from bacterial plasmids, bacteriophages, transposons, yeast episomes, insertion elements, yeast chromosomal elements, baculoviruses, papova viruses such as SV40, vaccinia viruses,  
15 adenoviruses, fowl pox viruses, pseudorabies viruses, picornaviruses and retroviruses and vectors derived from combinations thereof, such as cosmids and phagemids.

The vectors of the invention can be formulated in microparticles, with adjuvants, lipid, buffer or other  
20 excipients as appropriate for a particular application.

In one embodiment of the invention the vector is an expression vector. Expression vectors typically comprise nucleic acid sequence elements that can control, regulate, cause or permit expression of a polypeptide encoded by such a  
25 vector. Such elements may comprise transcriptional enhancer binding sites, RNA polymerase initiation sites, ribosome binding sites, and other sites that facilitate the expression of encoded polypeptides in a given expression system. Such expression systems may be cell-based, or cell-free systems  
30 well known in the art. Nucleic acid sequence elements and parent vector sequences suitable for use in the expression of encoded polypeptides are also well known in the art. An exemplary plasmid-derived expression vector useful for expression of the polypeptides of the invention comprises an  
35 *E. coli* origin of replication, an aph(3')-1a kanamycin resistance gene, HCMV immediate early promoter with intron A, a synthetic polyA sequence and a bovine growth hormone terminator. Another exemplary plasmid derived expression

vector comprises an *E. coli* origin of replication, an ant(4')-Ia kanamycin resistance gene, Rous sarcoma virus long terminal repeat sequences, HCMV immediate early promoter and an SV40 late polyA sequence.

- 5        Another embodiment of the invention is an isolated host cell comprising a vector of the invention. Representative host cell examples include Archaea cells; bacterial cells such as *Streptococci*, *Staphylococci*, *Enterococci*, *E. coli*, *Streptomyces*, cyanobacteria, *B. subtilis* and *S. aureus*;
- 10        fungal cells such as *Kluyveromyces*, *Saccharomyces*, Basidiomycete, *Candida albicans* or *Aspergillus*; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293, CV-1, Bowes melanoma and myeloma; and plant cells, such as gymnosperm or
- 15        angiosperm cells. The host cells in the methods of the invention may be provided as individual cells, or populations of cells. Populations of cells may comprise an isolated or cultured population of cells or cells present in a matrix such as a tissue.
- 20        Introduction of a polynucleotide, such as a vector, into a host cell can be effected by methods well known to those skilled in the art (Davis et al., Basic Methods in Molecular Biology, 2<sup>nd</sup> ed., Appleton & Lange, Norwalk, CT, 1994; Sambrook et al., Molecular Cloning: A Laboratory Manual, 3<sup>rd</sup>
- 25        ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001). These methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic
- 30        introduction and infection.

- Another embodiment of the invention is an isolated polypeptide comprising a polypeptide having a sequence shown in SEQ ID NO: 4. SEQ ID NO: 4 is a polypeptide comprising variant human MDM2 protein with a D300A substitution. The
- 35        polypeptides of the invention may be produced by chemical synthesis, such as solid phase peptide synthesis, on an automated peptide synthesizer. Alternatively, the polypeptides of the invention can be obtained from

polynucleotides encoding these polypeptides by the use of cell-free expression systems such as reticulocyte lysate based expression systems, wheat germ extract based expression systems, and *Escherichia coli* extract based expression systems. The polypeptides of the invention can also be obtained by expression and isolation from cells harboring a nucleic acid sequence of the invention by techniques well known in the art, such as recombinant expression of easily isolated affinity labeled polypeptides. Those skilled in the art will recognize other techniques for obtaining the polypeptides of the invention. The polypeptides of the invention may comprise fusion polypeptides comprising a polypeptide of the invention fused with a second polypeptide. Such second polypeptides may be leader or secretory signal sequences, a pre- or pro- or prepro-protein sequence, as well as naturally occurring, or partially synthetic sequences derived in part from a naturally occurring sequence or an entirely synthetic sequence.

Another embodiment of the invention is a method for expressing a polypeptide comprising the steps of providing a host cell of the invention; culturing the host cell under conditions sufficient for the expression of at least one polypeptide comprising the sequence shown in SEQ ID NO: 4.

Host cells can be cultured under any conditions suitable for maintaining or propagating a given type of host cell and sufficient for expressing a polypeptide. Culture conditions, media, and related methods sufficient for the expression of polypeptides are well known in the art. For example, many mammalian cell types can be aerobically cultured at 37°C using appropriately buffered DMEM media while bacterial, yeast and other cell types may be cultured at 37°C under appropriate atmospheric conditions in LB media.

In the methods of the invention the expression of a polypeptide can be confirmed using a variety of different techniques well known in the art. For example, expression of MDM2<sup>A300D</sup> can be confirmed by Western blot or assaying ability of MDM2<sup>A300D</sup> to inhibit caspases.

The present invention will now be described with reference to the following specific, non-limiting examples.

### Examples

5 In the following Examples, CHO cell lines over-expressing apoptotic<sup>R</sup> genes were analyzed in shake flask cultures for peak viable cell density, longevity, caspase 3/7 activation, and improved production of secreted proteins.

### 10 Materials and Methods:

#### Cell culture:

CHOK1SV cell line (Lonza Biologics, Slough, UK), designated as the Control cell line C1013A, and CHOK1  
15 (American Type Culture Collection, Manassas, USA), designated as the Control cell line C1835 were cultured in CD-CHO medium (Cat. No. 10743-011, Invitrogen, Carlsbad, CA), containing 30 mM Glucose and supplemented with 6mM L-Glutamine (Invitrogen Cat. No. 10313-021). In some instances, another animal  
20 protein-free medium containing various concentrations including 60mM glucose (defined as the high glucose medium) was used. Fetal Bovine serum was purchased from Hyclone Labs, Logan, UT (Cat. No. SH30071.03). Cell cultures were monitored by a Cedex automated cell counting instrument  
25 (Innovatis, Germany). Integrated viable cell count (IVCC, cell-day/ml) was calculated using the following formula:

$$\text{IVCC (d1)} = [\text{VCD (d0)} + \text{VCD (d1)}] / 2 + \text{VCD (d0)},$$
where VCD = viable cell density

### 30 Expression vectors:

Coding sequence of Bcl-2Δ (SEQ ID NO: 11) was cloned under the CMV promoter into the pCDNA<sup>TM</sup>3.1(+) vector having Neo®. Coding sequence of Bcl-XL (SEQ ID NO: 9) was cloned under the CMV promoter into the pCDNA<sup>TM</sup>3.1(+) vector having  
35 Zeo®. Coding sequence of MDM2 (SEQ ID NO: 3) was cloned under the CMV promoter into the pCDNA<sup>TM</sup>3.1(+) vector having Neo®. pBUDCE4.1 vector designed to constitutively express E1B-19K (EF-1a promoter), either alone or in conjunction with

Aven (CMV promoter) has been described (Nivitchanyong et al.,  
Biotechnol. Bioeng. 98:825-841 (2007)). The vector  
expressing XIAPA (CMV promoter) has been described (Sauerwald  
et al., Biotechnol. Bioeng. 77:704-716 (2002)). MDM2<sup>D300A</sup>  
5 expression vector was generated by in vitro mutagenesis from  
the MDM2 expression vector. A model antibody (Ab #1)  
expression vector was constructed by cloning a heavy and a  
light chain cDNA into a Glutamine Synthase (GS) expression  
vector (obtained from Lonza Biologicals, Slough, UK, under a  
10 research license).

**Generation of apoptotic<sup>R</sup> cell lines:**

An exponential culture of CHOK1SV cell line was  
transfected with various combinations of expression vectors  
15 as shown in Table 1. Transfectomas were selected using a  
combination of 400 µg/ml hygromycin, 400 µg/ml gentamicin, or  
300 µg/ml Zeocin. About 200 resulting transfectomas were  
expanded into 24-well plate and caspase 3/7 activity  
determined by APO-ONE assay (Promega, Madison, WI). Two  
20 measurements were performed; a) early in growth phase (~day 3  
post-seeding) following treatment with Staurosporine to  
induce apoptosis; and b) late in growth phase (~day 10 post-  
seeding), at which time, a subset of wild-type cells has  
progressed into apoptosis. Transfectomas that had reduced  
25 caspase 3/7 activities in both cases were further expanded  
and top two to four clones were subjected to shake-flask  
batch growth profile studies.

Promising cell lines were cryopreserved. Shake-flask  
cultures of selected lines were tested for reduced caspase 3  
30 activity by FLOW, using fluorescent-labeled antibodies  
specific for caspase 3 (BD Bioscience; Cat. #68652X/550557).  
Selected cell lines were C-coded and submitted for cell  
banking. These cell lines underwent ten to 15 passage  
stability testing in the absence of antibiotics, which were  
35 used as selection agents. The cell lines generated are shown  
in Table 1. Expression of each transgene was confirmed in a  
select set of cell lines by Western blot.

**Table 1.**

| Cell line                    | Over-expressed gene                     |
|------------------------------|-----------------------------------------|
| B-31                         | Bcl-2 $\Delta$                          |
| BX-61                        | Bcl-2 $\Delta$ and XIAP $\Delta$        |
| BMX-13 and BMX-39            | Bcl-2 $\Delta$ , MDM2 and XIAP $\Delta$ |
| Bx-51                        | Bcl-XL                                  |
| BxMX-01, BxMX-11 and BxMX-25 | Bcl-XL, MDM2 and XIAP $\Delta$          |
| EM-15 and EM-70              | E1B19K and MDM2                         |
| EAX-197                      | E1B19K, AVEN and XIAP $\Delta$          |
| EA-167                       | E1B19K and AVEN                         |
| C1013A                       | none                                    |
| BM                           | MDM2 and Bcl-2 $\Delta$ ;               |
| BxM                          | MDM2, Bcl-XL                            |
| EMX-                         | MDM2, E1B19K, XIAP $\Delta$             |

5

**Shake-flask cultures of apoptotic<sup>R</sup> cell lines:**

Selected apoptotic<sup>R</sup> cell lines were cultured in batch mode in CD-CHO medium supplemented with 6mM Glutamine and the requisite antibiotic selection agent(s). CD-CHO medium is formulated with 30 mM glucose. Additionally, select Ab-expressing cell lines were cultured in a custom formulated animal protein-free medium supplemented with 6mM glutamine and 60mM glucose.

15

**Caspase 3/7 activity Assay:**

About  $3 \times 10^5$  cells of each clone were seeded in one ml of growth medium in a 24-well plate. On day 4 (d4) post seeding, about  $1 \times 10^5$  cells were transferred in triplicate to a 96well plate. Staurosporine (2 $\mu$ M fc) was added and the cells were incubated for 16h before assaying for caspase3/7 activity by APO-ONE kit (BD Labs). The procedure was

20

repeated on d10, except that Staurosporine was omitted. The clones that had significantly lower caspase3/7 activity on both days were expanded into shake flasks. The apoptotic<sup>R</sup> nature of the selected clones was confirmed by flow cytometry analysis (see below).

**Analysis of apoptotic<sup>R</sup> clones by flow cytometry:**

About  $1 \times 10^6$  cells from exponential cultures were withdrawn from each shake flask into 24well plates, incubated with Staurosporine (2 $\mu$ M fc) for 16h, harvested and washed once in PBS. The cells were then incubated with CytoPerm (Cat. No. 2075KK, BD BioScience) to fix and permeablized them. Following a PBS wash, cells were incubated with FITC-labeled anti-caspase3 (Cat. No. 68654, BD BioScience) antibody before subjecting them to analysis by flow cytometry.

**Example 1**

**Effect of MDM2 on Bcl-2 $\Delta$  expressing cell lines**

Growth profiles (SF/Batch) of BMX-13 and BMX-39, expressing Bcl-2 $\Delta$ , MDM2 and XIAP $\Delta$ , as well as double transfected cell line BX-61 expressing Bcl-2 $\Delta$  and XIAP $\Delta$ , B-31 expressing Bcl-2 $\Delta$  alone, and the control C1013A were analyzed. The peak viable cell count (VCD) of the control cell line reached  $6 \times 10^6$  cells/ml whereas those of BMX clones reached about  $11 \times 10^6$  cells/ml. Cell lines expressing Bcl-2 $\Delta$  and/or XIAP $\Delta$  had intermediate VCD (Figure 1A). BMX clones had higher integrated viable cell count (IVCC) compared to B-31 or BX-61. BMX-39 had a 44% increase in IVCC over control as compared to 23% for B-31. XIAP $\Delta$  had no incremental effect on IVCC when used in conjunction with Bcl-2 $\Delta$  (Figure 1B). High IVCC correlates with high viability of cells in the long term culture in a bioreactor, resulting in increased product yields. Further, biopharmaceuticals generated from a production cell line derived from an apoptotic<sup>R</sup> host cell line may be of superior quality. Cell lines BMX-13 and BMX-39 demonstrated caspase 3/7 downregulation by 10-fold and 16-fold, respectively when

compared to the control C1013A, confirming anti-apoptotic effect of the genes in the CHO cell line. B-31 and BX-61 had had 12- and 6-fold downregulation of caspases.

5

### Example 2

#### Effect of MDM2 on Bcl-XL expressing cell lines

Growth profiles of triple transfected BxMX-01, BxMX11 and BxMX-25, expressing Bcl-XL, MDM2 and XIAPΔ in comparison to Bx-51 expressing Bcl-XL alone as well as the control cell line C1013 were evaluated. The peak viable cell count (VCD) of control cell line reached 6x10<sup>6</sup> cells/ml whereas those of BxMX clones reached about 12x10<sup>6</sup> cells/ml (Figure 2A). Cell lines expressing Bcl-XL only had intermediate VCD. For example, the peak VCD of cell line Bx-51 was 10x10<sup>6</sup> cells/ml. BxMX clones had higher IVCC compared to Bx-51 with 34% increase in IVCC over control as compared to 18% for Bx-51 (Figure 2B). Co-transfection of Bcl-XL and MDM2 only (without XIAPΔ) failed to generate cell lines with higher VCD or extended longevity (data not shown). Thus, XIAPΔ and MDM2 are likely to function synergistically towards achieving the high IVCC observed in BxMX-01, BxMX-11 and BxMX-25 cell lines. Caspase 3/7 activity was down-regulated 7-, 5-, and 8-fold in BxMX-01, BxMX-11 and BxMX-25, respectively, as compared to control C1013A.

25

### Example 3

#### Effect of MDM2 on E1B19K expressing cell lines

Growth profiles of EM-15 and EM-70 expressing E1B19K and MDM2 are shown in Figure 3. For comparison, in a separate experiment, cell lines expressing E1B19K and AVEN (EA-167), or expressing E1B19K, AVEN and XIAPΔ (EAX-197) as well as the transfection host cell line, C1013A were included. The peak viable cell density (VCD) of control cell line reached 6x10<sup>6</sup> cells/ml whereas those of EM clones reached about 12x10<sup>6</sup> cells/ml to 16x10<sup>6</sup> cells/ml. The maximum VCD for EA-167 and EAX-197 was 13.6 - 13.9x10<sup>6</sup> cells/ml. The EM clones had higher IVCC compared to EA-167 or EAX-197. EM-70 had a 100% increase in IVCC over control as compared to 23% increase

- over control for EA-167. This data, along with the fact that cell lines expressing E1B19K alone were not sufficient for achieving the high IVCC observed in this experiment (Nivitchanyong et al., Biotechnol Bioeng 98:825-841 (2007))
- 5 suggests that MDM2 contributed to the increase in IVCC observed in EM-15 and EM-70 cell lines. Co-transfection of E1B19K, MDM2 and XIAP $\Delta$  failed to generate cell lines with higher VCD or IVCC as compared to (data not shown). Caspase 3/7 activity in EM-15 and EM-70 cell lines were 13% and 30%,
- 10 respectively, from the control C1013A. For comparison, EA-167 and EAX-197 had 37% and 20% of caspase 3/7 activity as that of C1013A control. Apoptosis was also confirmed by FLOW as described above. 91% of control cells were positive for caspase 3/7 whereas 1 - 30% of cells within cell lines
- 15 expressing apoptotic<sup>R</sup> genes were caspase 3/7 positive. Cell lines with the lowest caspase 3/7 activity (eg. B-31) did not necessarily have the highest IVCC.

#### Example 4

##### Cloning and expression of mutant MDM2

The vector expressing wild-type human MDM2 full-length cDNA (Genbank Accession M92424.1) was obtained from John  
5 Hopkins University. The MDM2<sup>D300A</sup> expression vector was generated by in vitro mutagenesis with a mutagenesis primer  
5' gctgaagagggcttt gatgtgccggcttgt aaaaaaactatagtg 3' (SEQ ID NO: 15, resulting in a replacement of A at position 899 with C, and in substitution of Aspartic acid for Alanine in the  
10 predicted MDM2<sup>D300A</sup> protein. The mutagenesis was confirmed by sequencing. The MDM2<sup>D300A</sup> DNA sequence is shown in SEQ ID NO: 3 and the predicted MDM2<sup>D300A</sup> protein sequence is shown in SEQ ID NO: 4. This new mutant vector as well as its wild-type counterpart was used for transient and stable transfections.  
15 The MDM2<sup>D300A</sup> and MDM2 wild type proteins were transiently expressed in Hek293 cells. Western blot demonstrated presence of higher levels of MDM2<sup>D300A</sup> in the cells when compared to the wild type MDM2, suggesting the mutant protein was more resistant to proteolytic degradation than the wild  
20 type MDM2.

#### Example 5

##### Generation of MDM2<sup>D300A</sup>-expressing cell lines

Stable cell lines over-expressing MDM2<sup>D300A</sup> or WT MDM2  
25 proteins were generated as described in Example 1. Two host cell lines, C1013A and C1835A were used. The list of cell lines used in growth profile studies is shown in Table 2.

Table 2.

| Cell line | Host   | Transfected genes            |
|-----------|--------|------------------------------|
| A3        | C1013A | WT MDM2                      |
| A4        | C1013A | WT MDM2                      |
| B1        | C1013A | MDM2D300A                    |
| B5        | C1013A | MDM2D300A                    |
| C7        | C1835A | WT MDM2                      |
| C8        | C1835A | WT MDM2                      |
| D6        | C1835A | MDM2D300A                    |
| D7        | C1835A | MDM2D300A                    |
|           | C1013A | Pool of wild-type MDM2 cells |
|           | C1013A | Pool of MDM2A300D cells      |
|           | C1013A | none                         |
|           | C1835A | none                         |
|           |        |                              |
| EM70      | C1013A | E1B19K , MDM2                |
| C1013H    | C1013A | Bcl2d                        |
| C1013J    | C1013A | Bcl-XL                       |
| C1013K    | C1013A | E1B19K, Aven , XIAPd         |
| BMX13     | C1013A | Bcl-2d, MDM2 , XIAPd         |

### Example 6

5 Effect of MDM2 on longevity and viability of CHOK1 host cell  
lines

The growth profiles and viability (Shake-Flask/Batch) of C1013A-derived and C1835A-derived cell lines D6, B1 and B5 expressing the MDM2D300A gene are shown in Figure 4. Using Centocor proprietary protein-free medium, the peak viable cell density (VCD) of C1013A control cell line was  $8 \times 10^6$  cells/ml, and that of the C1835A control cell line was  $5 \times 10^6$  cells/mL. Cell lines over-expressing MDM2<sup>D300A</sup> had increased longevity as compared to the control cell lines. In fed-batch cultures, B1 and B5 cell lines were maintained in culture for up to 20 days, whereas the untransfected host cell as well as the cells derived from bulk selected pool following transfection with MDM2<sup>D300A</sup> lost viability by day 14 of the culture (Figure 5).

Example 7Stability of the CHO cell lines over-expressing MDM2

The C1013A-derived and C1835A-derived CHO cell lines D6 and B5 over-expressing MDM2D300A were subjected to a 15-passage stability study, with and without the selection agent, genitacin. A growth curve study was conducted at the beginning and the end of the stability study for each cell line and peak viable cell density (indicative of cell line stability) was noted. The cultures without genitacin selection and at higher passages had equivalent or higher VCD's and thus can be considered to very stable in the absence of the selection reagent (Table 3).

Table 3: Peak VCD of Cell Lines Over-expressing MDM2.m (15-passage Stability Study)

|                              | Peak VCD (10 <sup>6</sup> /mL) |
|------------------------------|--------------------------------|
| MUT - B5 (-) genitacin (p1)  | 6.7                            |
| MUT - B5 (+) genitacin (p1)  | 6.4                            |
| MUT - B5 (-) genitacin (p15) | 9.1                            |
| MUT - B5 (+) genitacin (p15) | 7.3                            |
|                              |                                |
| MUT - D6 (-) genitacin (p1)  | 4.6                            |
| MUT - D6 (+) genitacin (p1)  | 4.5                            |
| MUT - D6 (-) genitacin (p15) | 4.6                            |
| MUT - D6 (+) genitacin (p15) | 5.4                            |

Example 8

Productivity Studies Using MDM2 Over-expressing Host Cell Lines.

The cell line, A4, over-expressing MDM2 and B1, over-expressing MDM2<sup>D300A</sup> were transfected with a recombinant antibody heavy and light chain expression vector. The transfection mixture was first bulk selected using glutamine-free media containing Glutamine Synthetase supplements and 25  $\mu$ M MSX. Subsequently, the mixture was plated in methocult for isolation of individual clones. About 100 resulting

transfectomas per transfection were expanded into 24-well plate and a 14-day spent titer was measured by nephelometry. The average titers for the MDM2 and MDM2D300A cell lines expressing CNTO328 were 90 mg/L, and significantly higher than that of clones derived from C1013A, which was 21.3 mg/L.

In a separate experiment, CHOK1SV cells were transfected with MDM2 and E1B19K and one clone, EM70 stably expressing E1B19K and MDM2 was transfected with a recombinant heavy and light chain antibody expression vector (Dorai et al., Biotechnol. Bioeng., 103:592-608 (2009)). The transfection mixture was first bulk selected using glutamine-free media containing Glutamine Synthetase supplements and 25uM MSX. For comparison, several other cell lines including C1013A (control), C1013M, C1013J, C1013K, A4, B5, BMX13. Following bulk selection for 29days, the surviving cells were subjected to a shake-flask fed-batch study. EM70 provided antibody titers of >700 mg/L on d23 whereas the titers of the remaining cell lines did not exceed 100 mg/L (Figure 6). An exponential culture of CHOK1SV was transfected with vectors expressing E1B19K and MDM2. Two days later antibiotic selection protocol was initiated. By day 29, all untransfected cells were eliminated whereas the antibiotic resistant cells (transfected pool) had survived.

These cells were used for performing a shake-flask fed-batch growth profile study. 2e5cells/ml was seeded in Mach-1 medium containing supplements. Starting day-2, the cultures were fed daily a nutrient mix of glucose and amino acids. Cell counts and titer was measured daily.

The present 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.

## Claims

1. A method of increasing production of a secreted protein in a Chinese Hamster Ovary (CHO) fed batch cell culture, comprising culturing a CHO cell line over-expressing MDM2 and E1B19K and one or more genes encoding the secreted protein
2. The method of claim 1 wherein the titer of the secreted protein is at least 600 mg/L.
3. The method of claim 1 wherein the CHO cell line is CHO-K1.
4. The method of claim 1 wherein the CHO cell line is CHO-K1SV.
5. The method of claim 1 wherein the secreted protein is an antibody heavy chain and an antibody light chain.

## SEQUENCE LISTING

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<120> USE OF THE ANTI-APOPTOTIC GENE HUMAN-MURINE DOUBLE  
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LINES EXPRESSING THERAPEUTIC PROTEINS

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 Ile Phe Glu Leu Lys Ser Val Ala Ala Gly Cys Pro Val Leu Leu Gly  
 245            250            255  
 Lys Asp Asn Pro Ser Pro Gly Pro Ser Arg Asp Ser Gln Lys Pro Thr  
 260            265            270  
 Ser Pro Leu Gln Ser Ala Gly Asp His Leu Glu Glu Glu Leu Asp Leu  
 275            280            285  
 Leu Leu Asn Leu Asp Ala Pro Ile Lys Glu Gly Asp Asn Ile Leu Pro  
 290            295            300  
 Asp Gln Thr Ser Gln Asp Leu Lys Ser Lys Glu Asp Gly Glu Val Val  
 305            310            315            320  
 Gln Glu Glu Glu Val Cys Ala Lys Pro Ser Val Thr Glu Glu Lys Asn  
 325            330            335  
 Met Glu Pro Glu Gln Pro Ser Thr Ser Lys Asn Val Thr Glu Glu Glu  
 340            345            350  
 Leu Glu Asp Trp Leu Asp Ser Met Ile Ser  
 355            360

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<211> 513

<212> DNA

<213> Homo sapiens

<400> 9

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 actgaatcgg agatggagac cccagtgcc atcaatggca accatcctg gcacctggca 180

gacagcccccg cgggtgaatgg agccactggc cacagcagca gtttgatgc ccgggaggtg 240  
 atcccatgg cagcagtaaa gcaagcgtg agggaggcag gcgacgagtt tgaactgcgg 300  
 taccggcggg cattcagtga cctgacatcc cagctccaca tcacccagg gacagcatat 360  
 cagagcttfg aacaggatac tttgtggaa ctctatggga acaatgcagc agccgagagc 420  
 cgaaagggcc aggaacgctt caaccgctgg ttctgacgg gcatgactgt ggccggcgtg 480  
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<211> 233

<212> PRT

<213> Homo sapiens

<400> 10

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 Asn Arg Thr Glu Ala Pro Glu Gly Thr Glu Ser Glu Met Glu Thr Pro  
 35 40 45  
 Ser Ala Ile Asn Gly Asn Pro Ser Trp His Leu Ala Asp Ser Pro Ala  
 50 55 60  
 Val Asn Gly Ala Thr Gly His Ser Ser Ser Leu Asp Ala Arg Glu Val  
 65 70 75 80  
 Ile Pro Met Ala Ala Val Lys Gln Ala Leu Arg Glu Ala Gly Asp Glu  
 85 90 95  
 Phe Glu Leu Arg Tyr Arg Arg Ala Phe Ser Asp Leu Thr Ser Gln Leu  
 100 105 110  
 His Ile Thr Pro Gly Thr Ala Tyr Gln Ser Phe Glu Gln Val Val Asn  
 115 120 125  
 Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe  
 130 135 140  
 Ser Phe Gly Gly Ala Leu Cys Val Glu Ser Val Asp Lys Glu Met Gln  
 145 150 155 160  
 Val Leu Val Ser Arg Ile Ala Ala Trp Met Ala Thr Tyr Leu Asn Asp  
 165 170 175  
 His Leu Glu Pro Trp Ile Gln Glu Asn Gly Gly Trp Asp Thr Phe Val  
 180 185 190  
 Glu Leu Tyr Gly Asn Asn Ala Ala Ala Glu Ser Arg Lys Gly Gln Glu  
 195 200 205  
 Arg Phe Asn Arg Trp Phe Leu Thr Gly Met Thr Val Ala Gly Val Val  
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 Leu Leu Gly Ser Leu Phe Ser Arg Lys  
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<210> 11

<211> 573

<212> DNA

<213> Homo sapiens

<400> 11

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ccacctgtgg tccacctgac cctccgccag gccggcgacg acttctccg ccgtaccgc 180
cgcgactteg ccgagatgtc cagccagctg cacctgacgc ccttcaccgc gcggggacgc 240
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caggataacg gaggtgggga tgcctttgtg gaactgtacg gcccagcat gcggcctctg 480
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<210> 12

<211> 190

<212> PRT

<213> Homo sapiens

<400> 12

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Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr Leu
 35     40     45
Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala
 50     55     60
Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg
 65     70     75     80
Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly
 85     90     95
Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser
100    105    110
Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met
115    120    125
Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly
130    135    140
Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu
145    150    155    160
Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala Leu
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Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
180    185    190

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<210> 13  
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 cctgtttcag catcaacact ggcacgagca ggggtttctt atactgggtga aggagatacc 180  
 gtgcgggtgct ttagtgtca tgcagctgta gatagatggc aatatggaga ctcagcagtt 240  
 ggaagacaca ggaaagtatc cccaaattgc agatttatca acggctttta tcttgaaaat 300  
 agtgccacgc agtctacaaa ttctgggtatc cagaatggtc agtacaaagt tgaaaactat 360  
 ctgggaagca gagatcattt tgccttagac aggccatctg agacacatgc agactatctt 420  
 ttgagaactg ggcagggtgt agatatatca gacaccatat acccgaggaa ccttgccatg 480  
 tatagtgaag aagctagatt aaagtccttt cagaactggc cagactatgc tcacctaacc 540  
 ccaagagagt tagcaagtgc tggactctac tacacaggta ttggtgacca agtgcagtcg 600  
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 aggcgacact ttctaattg cttctttgtt ttgggccgga atcttaatat tgaagtga 720  
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 tccatggcag attatgaagc acggatcttt acttttggga catggatata ctcagttaac 840  
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 aatattcatt taactcattc acttgaggag tgtctggtaa gaactactga gaaaacacca 1080  
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 cgaatggggg tcaagttcaa ggacattaag aaaataatgg aggaaaaaat tcagatatct 1200  
 gggagcaact ataatcact tgaggttctg gttgcagatc tagtgaatgc tcagaaagac 1260  
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 <212> PRT  
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 Phe Ala Asn Phe Pro Ser Gly Ser Pro Val Ser Ala Ser Thr Leu Ala  
 35 40 45  
 Arg Ala Gly Phe Leu Tyr Thr Gly Glu Gly Asp Thr Val Arg Cys Phe  
 50 55 60

Ser Cys His Ala Ala Val Asp Arg Trp Gln Tyr Gly Asp Ser Ala Val  
 65                70                75                80  
 Gly Arg His Arg Lys Val Ser Pro Asn Cys Arg Phe Ile Asn Gly Phe  
                  85                90                95  
 Tyr Leu Glu Asn Ser Ala Thr Gln Ser Thr Asn Ser Gly Ile Gln Asn  
                  100                105                110  
 Gly Gln Tyr Lys Val Glu Asn Tyr Leu Gly Ser Arg Asp His Phe Ala  
                  115                120                125  
 Leu Asp Arg Pro Ser Glu Thr His Ala Asp Tyr Leu Leu Arg Thr Gly  
                  130                135                140  
 Gln Val Val Asp Ile Ser Asp Thr Ile Tyr Pro Arg Asn Pro Ala Met  
 145                150                155                160  
 Tyr Ser Glu Glu Ala Arg Leu Lys Ser Phe Gln Asn Trp Pro Asp Tyr  
                  165                170                175  
 Ala His Leu Thr Pro Arg Glu Leu Ala Ser Ala Gly Leu Tyr Tyr Thr  
                  180                185                190  
 Gly Ile Gly Asp Gln Val Gln Cys Phe Cys Cys Gly Gly Lys Leu Lys  
                  195                200                205  
 Asn Trp Glu Pro Cys Asp Arg Ala Trp Ser Glu His Arg Arg His Phe  
                  210                215                220  
 Pro Asn Cys Phe Phe Val Leu Gly Arg Asn Leu Asn Ile Arg Ser Glu  
 225                230                235                240  
 Ser Asp Ala Val Ser Ser Asp Arg Asn Phe Pro Asn Ser Thr Asn Leu  
                  245                250                255  
 Pro Arg Asn Pro Ser Met Ala Asp Tyr Glu Ala Arg Ile Phe Thr Phe  
                  260                265                270  
 Gly Thr Trp Ile Tyr Ser Val Asn Lys Glu Gln Leu Ala Arg Ala Gly  
                  275                280                285  
 Phe Tyr Ala Leu Gly Glu Gly Asp Lys Val Lys Cys Phe His Cys Gly  
                  290                295                300  
 Gly Gly Leu Thr Asp Trp Lys Pro Ser Glu Asp Pro Trp Glu Gln His  
 305                310                315                320  
  
 Ala Lys Trp Tyr Pro Gly Cys Lys Tyr Leu Leu Glu Gln Lys Gly Gln  
                  325                330                335  
 Glu Tyr Ile Asn Asn Ile His Leu Thr His Ser Leu Glu Glu Cys Leu  
                  340                345                350  
 Val Arg Thr Thr Glu Lys Thr Pro Ser Leu Thr Arg Arg Ile Asp Asp  
                  355                360                365  
 Thr Ile Phe Gln Asn Pro Met Val Gln Glu Ala Ile Arg Met Gly Phe  
                  370                375                380  
 Ser Phe Lys Asp Ile Lys Lys Ile Met Glu Glu Lys Ile Gln Ile Ser  
 385                390                395                400  
 Gly Ser Asn Tyr Lys Ser Leu Glu Val Leu Val Ala Asp Leu Val Asn  
                  405                410                415  
 Ala Gln Lys Asp Ser Met Pro Asp Glu Ser Ser Gln Thr Ser Leu Gln

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<211> 45

<212> DNA

<213> Homo sapiens

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