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(54) **RECOMBINANT PROTEIN PRODUCTION**

HERSTELLUNG REKOMBINANTER PROTEINE

PRODUCTION DE PROTÉINES RECOMBINANTES

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(56) References cited:  
**EP-A1- 1 911 840 EP-A1- 2 246 428**

- **M. JOHANSSON ET AL: "Genetic code translation displays a linear trade-off between efficiency and accuracy of tRNA selection", PROCEEDINGS NATIONAL ACADEMY OF SCIENCES PNAS, vol. 109, no. 1, 3 January 2012 (2012-01-03), pages 131-136, XP055352134, US ISSN: 0027-8424, DOI: 10.1073/pnas.1116480109**
- **J. R. IBEN ET AL: "tRNAomics: tRNA gene copy number variation and codon use provide bioinformatic evidence of a new anticodon:codon wobble pair in a eukaryote", RNA, vol. 18, no. 7, 14 May 2012 (2012-05-14), pages 1358-1372, XP055352434, US ISSN: 1355-8382, DOI: 10.1261/rna.032151.111**

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- **HERVÉ SELIGMANN: "Error compensation of tRNA misacylation by codon-anticodon mismatch prevents translational amino acid misinsertion", COMPUTATIONAL BIOLOGY AND CHEMISTRY, vol. 35, no. 2, 1 April 2011 (2011-04-01), pages 81-95, XP055352466, AMSTERDAM, NL ISSN: 1476-9271, DOI: 10.1016/j.compbiolchem.2011.03.001**
- **N. WALD ET AL: "Codon usage bias in prokaryotic pyrimidine-ending codons is associated with the degeneracy of the encoded amino acids", NUCLEIC ACIDS RESEARCH, vol. 40, no. 15, 11 May 2012 (2012-05-11), pages 7074-7083, XP055352443, ISSN: 0305-1048, DOI: 10.1093/nar/gks348**
- **AGRIS P F ET AL: "tRNA's Wobble Decoding of the Genome: 40 Years of Modification", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 366, no. 1, 9 February 2007 (2007-02-09), pages 1-13, XP027064727, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2006.11.046 [retrieved on 2007-01-23]**

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**Description****Field of the Invention**

5 **[0001]** The disclosure relates to recombinant protein expression systems comprising genetically modified cells, excluding human germ cells, wherein the cells are transformed or transfected with tRNA genes to reduce base mismatch due to genetic degeneracy in the genetic code and hence reliance on wobble for decoding; the modified cells have so improved recombinant protein expression.

**Background to the Invention**

10 **[0002]** The amino acid composition of proteins determines their properties and is encoded by the DNA sequence of genes. The gene sequence is copied into messenger RNA (mRNA) and decoded using transfer RNA (tRNA) that delivers amino acids in the specified order. The code is written in contiguous triplets of bases, called codons and is decoded by binding to the tRNAs' complementary base triplets, called anticodons. Each tRNA has a single anticodon and delivers a specified amino acid. However, although the combination of the four bases present in the DNA provides 64 codons, there are fewer tRNAs and anticodons present.

15 **[0003]** DNA comprises four different bases whereby adenosine specifically binds with thymine and guanine specifically binds with cytosine. All organisms have fewer species of tRNA than codons and therefore some tRNA species must pair with more than one codon, which led to the "Wobble" hypothesis postulating that the 5' base on the anticodon, which binds to the 3' base on the mRNA, can have non-standard base pairing, with only two of the three bases optimally matched. Suboptimal base pairing can lead to misincorporation errors upon translation. The most frequent errors correspond to a G (mRNA)/U(tRNA) base pair mismatch and additional wobble position mismatches (C/U and U/U) during translation.

20 **[0004]** Heterologous expression of recombinant proteins provided a wealth of diagnostic and therapeutic agents. Often bacterial systems are the preferred host for protein expression due to the low maintenance and easy handling. However, inability to accommodate for posttranslational modifications led to the usage of other expression systems such as yeast, insect cells, plants or mammalian expression systems. Approximately 70% of recombinant protein pharmaceuticals and most proteins used for vaccination, human therapy or diagnostics are currently produced in mammalian cells or cell lines, such as CHO or HEK293. However, although the choice of expression systems aids expression, there are still significant misincorporation levels being reported when expressing therapeutic monoclonal antibodies in CHO cells during clinical development. The impact of such sequence variation affects not just the quality and efficacy but could compromise patient safety by triggering an immunogenic response.

25 **[0005]** Optimal protein expression systems leading to error-free and soluble protein expression are highly desired. As described above, no organism harbours a full set of tRNAs and moreover, there are species-dependent differences. Expression of e.g. a human-derived transgene in a bacterial system is often problematic, as the codon usage and frequency can differ greatly, leading to inefficient expression. This problem is generally addressed by optimising codons to the preferred codon usage by the expression hosts. However, manipulating transgene sequence is extremely problematic, as it interferes with the structure, folding, stability and regulation of the encoded mRNA transcript in ways that are poorly understood and highly unpredictable. An alternative approach is to introduce additional tRNA transgenes into the host so as to improve codon recognition; this has been used successfully in *E. coli*, but has not been attempted in eukaryotic hosts. In addition, this approach does not deal with the problems associated with wobble such as inefficient translation and reduced translational fidelity.

30 **[0006]** WO2007/103307 discloses the incorporation of non-natural amino acids into proteins. WO2007/103307 discloses host cells that are modified to express modified tRNAs and modified aminoacyl-tRNA synthetases. WO2004/085463 discloses the incorporation of "unnatural amino acids" into proteins. US2005/287639 discloses the use of a modified aminoacyl-tRNA synthetase to charge "non-standard amino acids" onto modified tRNAs wherein the anticodon of said tRNA is also altered so that strict Watson and Crick base-pairings are formed. WO2014/160032 discloses a sequence 100% identical to the specific 7SL sequence. EP1911840 discloses a process of producing proteins comprising "unnatural amino acids" expressing an aminoacyl-tRNA synthetase. EP2246428 discloses the introduction of "non-canonical" (unnatural) amino acids into proteins and modified tRNAs to improve the aminoacylation of the tRNA with a non-natural amino acid. WO2004/024915 discloses gene expression systems based on codon translation efficiencies and wherein one codon is replaced with a codon of higher translation efficiency through introduction of iso-tRNAs in CHO cells. US2013/149699 discloses the modification of codons in mRNA to improve the translational efficiency by reducing the dependency on wobble to increase ribosome transit along a messenger RNA. Johansson et al (PNAS vol 109(1) p131-136, 2012) discloses an *in vitro* method for analysing codon usage by tRNAs to discover the parameters that are relevant to accuracy of tRNA recognition of codons in mRNAs. Iben et al (RNA vol 18(7) 1358-1372, 2012) discloses the correlation between tRNA abundance and codon recognition in non-complex organisms such as yeast.

Herve Seligmann (Computational Biology and Chemistry, vol 35(2), p81-95, 2011) discloses codon-anticodon mismatches and tRNA misloading leading to mis-insertion of amino acids. Wald et al (Nucleic Acids Research, vol 40(15), 2012) discloses degeneracy of the genetic code and codon usage bias in mRNA and how this relates to the population of tRNAs within a cell. WO2015/048989 is a systematic approach to codon selection for provision of a coding sequence for functional expression of a heterologous protein in a host cell.

**[0007]** The present disclosure relates to the introduction of tRNA genes with anticodons to optimally match codons that otherwise depend on wobble; in this way, unintended heterogeneity can be reduced and efficiency of protein synthesis can be improved.

## Statement of the Invention

**[0008]** According to an aspect of the invention there is provided an isolated eukaryotic cell, wherein said isolated eukaryotic cell is not a human germ cell, characterized in that said cell is modified by transfection or transformation with a nucleic acid molecule comprising a transcription cassette, the transcription cassette comprising at least one nucleotide sequence encoding a transfer RNA [tRNA],

the tRNA including an anticodon nucleotide sequence selected from the group consisting of AGC, GGC, CGC, UGC, ACC, GCC, CCC, UCC, AGG, GGG, CGG, UGG, AGU, GGU, CGU, UGU, AAC, GAC, CAC, UAC, AAA, GAA, AUU, GUU, CUU, UUU, AUC, GUC, CUC, UUC, AGA, GGA, CGA, UGA, ACU, GCU, ACG, GCG, CCG, UCG, CCU, UCU, AAG, GAG, CAG, UAG, CAA, UAA, AAU, GAU, UAU, CAU, AUA, GUA, ACA, GCA, AUG, GUG, CUG, UUG, CCA and

that is absent from said eukaryotic cell, wherein said anticodon sequence corrects base mismatches at any one of the cognate first, second or third nucleotide position in the corresponding messenger RNA [mRNA] codon to improve translation efficiency and/or decrease amino acid mis-incorporation as a consequence of degeneracy in the genetic code, and wherein said base mismatch correction reduces said cells reliance on wobble during translation of said mRNA.

**[0009]** "Wobble" is a well-known natural phenomenon. The genetic code means that a cell would need tRNAs with 61 different anticodons to complement the available 61 codons. However, due to the degeneracy of the genetic code, the third base is less discriminatory for the amino acid than the other two bases. This third position in the codon is referred to as the "wobble" position. At this position Us and Cs may be read by a G in the anticodon. Similarly, As and Gs may be read by a U or y (pseudouridine) in the anticodon, and A, U or C can be read by inosine in the anticodon. This flexibility in codon recognition can result in less efficient translation and potential mis-incorporation of amino acids. The invention addresses these problems by genetically engineering cells to reduce the reliance on "wobble" to enhance translation and increase translation fidelity. The transcription cassette according to the invention is adapted for expression of said tRNA. This includes the provision of a suitable RNA polymerase III promoter. Alternatively, the transcription cassette can be intergrated into the genome of said cell at a position near to a suitable promoter, for example an RNA polymerase III promoter.

**[0010]** In a preferred embodiment of the invention said nucleic acid molecule is part of an expression vector adapted for eukaryotic expression of said tRNA.

**[0011]** In a preferred embodiment of the invention said cell is further modified by transformation or transfection with a nucleic acid molecule encoding a recombinant protein, polypeptide or peptide.

**[0012]** In a preferred embodiment of the invention said expression vector is adapted for expression in a mammalian cell.

**[0013]** Typically, said adaptation includes the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive. "Promoter" is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only. Enhancer elements are *cis* acting nucleotide sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to *trans* acting transcription factors (polypeptides) which have been shown to bind specifically to enhancer elements. The binding/activity of transcription factors is responsive to a number of physiological/environmental cues which include, by example and not by way of limitation, intermediary metabolites (e.g. glucose, lipids), environmental effectors (e.g. heat). Promoter elements also include so called TATA box and RNA polymerase initiation selection sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, *inter alia*, to facilitate transcription initiation selection by RNA polymerase II or III. The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

**[0014]** Adaptations also include the provision of selectable markers and autonomous replication sequences which facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors. Episomal vectors are desirable since these molecules can incorporate large DNA fragments (30-50kb DNA). Episomal vectors of this type are described in WO98/07876. Alternatively, vectors can be "integrating vectors" which recombine with chromosomal DNA to stably transform the cell into which the integrating vector is incorporated.

**[0015]** Adaptations which facilitate the expression of vector encoded recombinant genes include the provision of transcription termination/polyadenylation sequences. This also includes the provision of internal ribosome entry sites (IRES) which function to maximise expression of vector encoded genes arranged in bi-cistronic or multi-cistronic expression cassettes. Expression control sequences also include so-called Locus Control Regions (LCRs). These are regulatory elements which confer position-independent, copy number-dependent expression to linked genes when assayed as transgenic constructs. LCRs include regulatory elements that insulate transgenes from the silencing effects of adjacent heterochromatin, Grosveld et al., *Cell* (1987), 51: 975-985. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) *DNA Cloning Techniques: A Practical Approach Vol III* IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

**[0016]** Additionally, a number of viruses are commonly used as vectors for the delivery of exogenous genes. Commonly employed vectors include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridae, parvoviridae, picornaviridae, herpesviridae, poxviridae, adenoviridae, or picornaviridae. Chimeric vectors may also be employed which exploit advantageous elements of each of the parent vector properties (See e.g., Feng, et al. (1997) *Nature Biotechnology* 15:866-870). Such viral vectors may be wild-type or may be modified by recombinant DNA techniques to be replication deficient, conditionally replicating or replication competent.

**[0017]** In a preferred embodiment of the invention said expression vector is adapted for expression in a fungal cell.

**[0018]** In a preferred embodiment of the invention said expression vector is adapted for expression in an insect cell.

**[0019]** In a preferred embodiment of the invention said expression vector is adapted for expression in a plant cell.

**[0020]** Suitable promoters for expression of recombinant proteins or tRNAs in plant cells include constitutive, tissue-specific, inducible, developmental or other promoters for expression in plant cells. Such promoters include viral, fungal, bacterial, animal and plant-derived promoters capable of functioning in plant cells. Constitutive promoters include, for example CaMV 35S promoter (Odell et al. (1985) *Nature* 313, 9810-812); rice actin (McElroy et al. (1990) *Plant Cell* 2: 163-171); ubiquitin (Christian et al. (1989) *Plant Mol. Biol.* 18 (675-689); pEMU (Last et al. (1991) *Theor Appl. Genet.* 81: 581-588); MAS (Velten et al. (1984) *EMBO J.* 3. 2723-2730); ALS promoter (U.S. Application Serial No. 08/409,297), and the like. Other constitutive promoters include those in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680, 5,268,463; and 5,608,142, Chemical-regulated plant promoters can be used to modulate the expression of a gene in a plant cell through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art of plant transgenic expression and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated plant promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena et al. (1991) *Proc. Natl. Acad. Sci. USA* 88: 10421-10425 and McNellis et al. (1998) *Plant J.* 14(2): 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz et al. (1991) *Mol. Gen. Genet.* 227: 229-237, and US Patent Nos. 5,814,618 and 5,789,156,

**[0021]** Where enhanced expression in particular tissues is desired, tissue-specific promoters can be utilised. Tissue-specific promoters include those described by Yamamoto et al. (1997) *Plant J.* 12(2): 255-265; Kawamata et al. (1997) *Plant Cell Physiol.* 38(7): 792-803; Hansen et al. (1997) *Mol. Gen. Genet.* 254(3): 337-343; Russell et al. (1997) *Transgenic Res.* 6(2): 157-168; Rinehart et al. (1996) *Plant Physiol.* 112(3): 1331-1341; Van Camp et al. (1996) *Plant Physiol.* 112(2): 525-535; Canevascni et al. (1996) *Plant Physiol.* 112(2): 513-524; Yamamoto et al. (1994) *Plant Cell Physiol.* 35(5): 773-778; Lam (1994) *Results Probl. Cell Differ.* 20: 181-196; Orozco et al. (1993) *Plant Mol. Biol.* 23(6): 1129-1138; Mutsuoka et al. (1993) *Proc. Natl. Acad. Sci. USA* 90 (20): 9586-9590; and Guevara-Garcia et al (1993) *Plant J.* 4(3): 495-50.

**[0022]** Particular of interest in the present context are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success in plants are described by Guerinneau and Mullineaux (1993) (*Plant transformation and expression vectors*. In: *Plant Molecular Biology Labfax* (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. Suitable vectors may include plant viral-derived vectors (see e.g. EP194809). If desired, selectable genetic markers may be included in the construct, such as those that confer selectable phenotypes such as

resistance to herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

**[0023]** In a preferred embodiment of the invention said isolated eukaryotic cell is an insect, plant, fungal or mammalian cell.

**[0024]** In a preferred embodiment of the invention said isolated mammalian cell is a non-human mammalian cell.

**[0025]** In a further preferred embodiment of the invention said isolated mammalian cell is selected from the group consisting of: Chinese Hamster Ovary, [CHO], HEK293, NS0 or CAP cells.

**[0026]** In a further preferred embodiment of the invention said fungal cell is selected from the group consisting of yeast: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* *Pichia pastoris*, *Aspergillus spp* [e.g. *A. niger*], *Kluyveromyces lactis* and *Trichoderma reesei* and *Yarrowia lipolytica*.

**[0027]** In a preferred embodiment of the invention said plant cell is isolated from a plant species selected from the group: corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa ssp.*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea spp.*), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus tree (*Citrus spp.*) cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa spp.*), avocado (*Persea americana*), fig (*Ficus carica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

**[0028]** Preferably, plant cells of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea, and other root, tuber or seed crops). Important seed crops are oil-seed rape, sugar beet, maize, sunflower, soybean, and sorghum. Horticultural plants to which the present invention may be applied may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower, and carnations and geraniums. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum. Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea.

**[0029]** In a further preferred embodiment of the invention said expression cassette comprises nucleotide sequences encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 different tRNAs.

**[0030]** In an embodiment of the invention said nucleic acid encodes a transfer RNA selected from the group of Ala-tRNA, Arg-tRNA, Asp-tRNA, Asn-tRNA, Cys-tRNA, Glu-tRNA, Gln-tRNA, Gly-tRNA, His-tRNA, Ile-tRNA, Leu-tRNA, Lys-tRNA, Met-tRNA, Phe-tRNA, Pro-tRNA, Ser-tRNA, Thr-tRNA, Trp-tRNA, Tyr-tRNA and Val-tRNA.

**[0031]** In a preferred embodiment said anticodon is cytosine and/or adenine rich.

**[0032]** In a preferred embodiment said anticodon is uracil free.

**[0033]** In a preferred embodiment of the invention said modified cell is transformed or transfected with a nucleic acid molecule encoding a therapeutic antibody.

**[0034]** In an alternative embodiment of the invention said modified cell is transformed or transfected with a nucleic acid molecule encoding a pharmaceutical or therapeutic protein or peptide.

**[0035]** In a further preferred embodiment of the invention said modified cell is transformed or transfected with a nucleic acid molecule encoding an industrial enzyme.

**[0036]** In a preferred embodiment of the invention said modified cell is further modified by transformation or transfection with a nucleic acid molecule comprising a 7SL nucleotide sequence.

**[0037]** Secreted and membrane proteins contain signal sequences that are required to target proteins to be secreted to the endoplasmic reticulum. The signal sequences of native and recombinant proteins destined for secretion interact with a signal recognition particle which is a ribonucleoprotein complex comprising six different proteins and a structural RNA of around 300 nucleotides and is referred to as 7SL RNA. 7SL RNA is conserved across species and although there is little homology at the level of sequence identity the secondary structures of 7SL RNA are highly conserved comprising a central double stranded region which is flanked at one end by two small stem loop structures and at the other end by two relatively large stem loop structures. We disclose that co-expression of 7SL nucleic acid in our modified cells greatly enhances expression of recombinant protein.

**[0038]** In a preferred embodiment of the invention said nucleic acid molecule comprises the nucleotide sequence set forth in SEQ ID NO: 10, or a nucleotide sequence variant that has at least 90% nucleotide sequence identity over the full length to the nucleotide sequence set forth in SEQ ID NO: 10.

**[0039]** In a preferred embodiment of the invention there is provided a nucleic acid molecule that comprises a nucleotide sequence that has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% nucleotide sequence identity over the full length to the nucleotide sequence set forth in SEQ ID NO: 10.

[0040] Preferably, said nucleic acid molecule comprises or consists of a nucleotide sequence as set forth in SEQ ID NO: 10.

[0041] In a preferred embodiment of the invention translation efficiency is enhanced by greater than 1-fold when compared to a non-modified cell of the same species.

5 [0042] In a preferred embodiment of the invention translation efficiency is enhanced at least two fold when compared to a non-modified cell of the same species.

[0043] Preferably, translational efficiency is enhanced at least 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold or at least 10-fold when compared to a non-modified cell of the same species. Preferably, translation is enhanced by greater than 10-fold when compared to a non-modified cell of the same species.

10 [0044] In a preferred embodiment of the invention the mis-incorporation of amino acids in said recombinant protein is suppressed by greater than 1-fold when compared to the amino acid mis-incorporation rate in a non-modified cell of the same species.

[0045] In a preferred embodiment of the invention the mis-incorporation of amino acids in said recombinant protein is suppressed by at least 2-fold when compared to the amino acid mis-incorporation rate in a non-modified cell of the same species.

15 [0046] Preferably, mis-incorporation is suppressed by at least 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 15-fold, 20-fold, 25-fold, 30-fold, 35-fold or at least 40-fold when compared to the amino acid mis-incorporation rate in a non-modified cell of the same species.

[0047] According to an aspect of the invention there is provided a modified cell according to the invention for use in reducing tRNA mismatch as a consequence of the degeneracy in the genetic code in the production of recombinant protein.

20 [0048] Preferably, said reduction in tRNA mismatch reduces said cell's reliance on wobble during translation of said mRNA by said modified cell.

[0049] According to a further aspect of the invention there is provided a method for reducing tRNA mismatch as a consequence of the degeneracy in the genetic code comprising the steps:

- 25
- i) providing a modified cell according to the invention;
  - ii) incubating the cell in i) under cell culture conditions suitable for expression of one or more recombinant proteins/peptides expressed by said modified cell; and optionally
  - 30 iii) isolating the expressed recombinant proteins/peptides from the modified cell or cell culture medium.

[0050] According to a further aspect of the invention there is provided a method to customise expression of a selected recombinant protein in a cell expression system comprising the steps:

- 35
- i) analysing the nucleotide sequence of a nucleic acid molecule encoding a recombinant protein to be expressed to identify one or more codon/anticodon tRNA mismatches;
  - ii) transfecting or transforming a cell with one or more transcription cassettes comprising one or more tRNA genes that correct at least one base pairing mismatch in at least one codon to provide a modified cell;
  - iii) transfecting or transforming said modified cell with the recombinant protein to be expressed; and optionally
  - 40 iv) providing cell culture conditions to express said recombinant protein.

[0051] In a preferred method of the invention said cell is a eukaryotic cell.

[0052] Preferably, said cell is a mammalian cell, a fungal cell, an insect cell or a plant cell.

[0053] In an alternative preferred method of the invention said cell is a prokaryotic cell, for example a bacterial cell.

45 [0054] According to an aspect of the invention there is provided a modified cell obtained or obtainable by the method according to the invention.

[0055] According to a further aspect of the invention there is provided a cell culture vessel comprising a modified cell according to the invention. In a preferred embodiment of the invention said cell culture vessel is a bioreactor such as a fermenter.

50 [0056] If eukaryotic microbial or prokaryotic expression is preferred in the methods according to the invention they are grown or cultured in the manner with which the skilled worker is familiar, depending on the host organism. As a rule, microorganisms are grown in a liquid medium comprising a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins, at temperatures of between

55 0°C and 100°C, preferably between 10°C and 60°C, while gassing in oxygen.

[0057] The pH of the liquid medium can either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batch wise, semi-batch wise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semi-continuously or continuously. Recombinant protein produced can be isolated from the

organisms as described above by processes known to the skilled worker, for example by extraction, if appropriate precipitation with salt, and/or chromatography. To this end, the organisms can advantageously be disrupted beforehand.

**[0058]** In this process, the pH value kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8. The culture medium to be used must suitably meet the requirements of the strains in question.

Descriptions of culture media for various microorganisms can be found in the textbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

**[0059]** As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements. Preferred carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media via complex compounds such as molasses or other by-products from sugar refining. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil, peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

**[0060]** Nitrogen sources are usually organic or inorganic nitrogen compounds or materials comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as cornsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a mixture.

**[0061]** Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron. Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine. Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

**[0062]** The fermentation media used according to the invention for culturing microorganisms usually also comprise other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, panthothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium.

**[0063]** Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps. "Consisting essentially" means having the essential integers but including integers which do not materially affect the function of the essential integers.

**[0064]** Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

**[0065]** Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

### Preferred Embodiments

**[0066]** Antibodies include polyclonal, monoclonal antibodies, humanised and chimeric antibodies and derived fragments comprising CDRs.

**[0067]** Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complementarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complementarity determining regions (CDRs) are the regions within the N-terminal domain of both the heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.

**[0068]** Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human

diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are therefore thought to be less immunogenic than chimeric antibodies.

**[0069]** Various fragments of antibodies are known in the art. A Fab fragment is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, covalently coupled together and capable of specifically binding to an antigen. Fab fragments are generated via proteolytic cleavage (with, for example, papain) of an intact immunoglobulin molecule. A Fab<sub>2</sub> fragment comprises two joined Fab fragments. When these two fragments are joined by the immunoglobulin hinge region, a F(ab')<sub>2</sub> fragment results. An Fv fragment is multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically binding to an antigen. A fragment could also be a single chain polypeptide containing only one light chain variable region, or a fragment thereof that contains the three CDRs of the light chain variable region, without an associated heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multi specific antibodies formed from antibody fragments, this has for example been described in US patent No 6,248,516. Fv fragments or single region (domain) fragments are typically generated by expression in host cell lines of the relevant identified regions. These and other immunoglobulin or antibody fragments are within the scope of the invention and are described in standard immunology textbooks such as Paul, Fundamental Immunology or Janeway et al. Immunobiology (cited above). Molecular biology now allows direct synthesis (via expression in cells or chemically) of these fragments, as well as synthesis of combinations thereof. A fragment of an antibody or immunoglobulin can also have bispecific function as described above.

### Pharmaceutical Proteins & Peptides

**[0070]** Examples of pharmaceutical proteins include "cytokines". Cytokines are involved in a number of diverse cellular functions. These include modulation of the immune system, regulation of energy metabolism and control of growth and development. Cytokines mediate their effects via receptors expressed at the cell surface on target cells. Examples of cytokines include the interleukins such as: IL1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32 and 33. Other examples include growth hormone, leptin, erythropoietin, prolactin, tumour necrosis factor [TNF] granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony stimulating factor (GM-CSF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF) and oncostatin M (OSM), interferon  $\alpha$ , interferon  $\beta$ , interferon  $\epsilon$ , interferon  $\kappa$  and  $\omega$  interferon.

**[0071]** Examples of pharmaceutically active peptides include GLP-1, anti-diuretic hormone; oxytocin; gonadotropin releasing hormone, corticotrophin releasing hormone; calcitonin, glucagon, amylin, A-type natriuretic hormone, B-type natriuretic hormone, ghrelin, neuropeptide Y, neuropeptide YY<sub>3-36</sub>, growth hormone releasing hormone, somatostatin; or homologues or analogues thereof.

**[0072]** The term "chemokine" refers to a group of structurally related low-molecular weight factors secreted by cells having mitogenic, chemotactic or inflammatory activities. They are primarily cationic proteins of 70 to 100 amino acid residues that share four conserved cysteine residues. These proteins can be sorted into two groups based on the spacing of the two amino-terminal cysteines. In the first group, the two cysteines are separated by a single residue (C-x-C), while in the second group they are adjacent (C-C). Examples of members of the 'C-x-C' chemokines include but are not limited to platelet factor 4 (PF4), platelet basic protein (PBP), interleukin-8 (IL-8), melanoma growth stimulatory activity protein (MGSA), macrophage inflammatory protein 2 (MIP-2), mouse Mig (m119), chicken 9E3 (or pCEF-4), pig alveolar macrophage chemotactic factors I and II (AMCF-I and -II), pre-B cell growth stimulating factor (PBSF), and IP10. Examples of members of the 'C-C' group include but are not limited to monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 2 (MCP-2), monocyte chemotactic protein 3 (MCP-3), monocyte chemotactic protein 4 (MCP-4), macrophage inflammatory protein 1  $\alpha$  (MIP-1- $\alpha$ ), macrophage inflammatory protein 1  $\beta$  (MIP-1- $\beta$ ), macrophage inflammatory protein 1- $\gamma$  (MIP-1- $\gamma$ ), macrophage inflammatory protein 3  $\alpha$  (MIP-3- $\alpha$ ), macrophage inflammatory protein 3  $\beta$  (MIP-3- $\beta$ ), chemokine (ELC), macrophage inflammatory protein-4 (MIP-4), macrophage inflammatory protein 5 (MIP-5), LD78  $\beta$ , RANTES, SIS-epsilon (p500), thymus and activation-regulated chemokine (TARC), eotaxin, I-309, human protein HCC-1/NCC-2, human protein HCC-3.

**[0073]** A number of growth factors have been identified which promote/activate endothelial cells to undergo angiogenesis. These include vascular endothelial growth factor (VEGF A); VEGF B, VEGF C, and VEGF D; transforming growth factor (TGF $\beta$ ); acidic and basic fibroblast growth factor (aFGF and bFGF); and platelet derived growth factor (PDGF). VEGF is an endothelial cell-specific growth factor which has a very specific site of action, namely the promotion of endothelial cell proliferation, migration and differentiation. VEGF is a complex comprising two identical 23 kD polypeptides. VEGF can exist as four distinct polypeptides of different molecular weight, each being derived from an alternatively spliced mRNA. bFGF is a growth factor that functions to stimulate the proliferation of fibroblasts and endothelial cells. bFGF is a single polypeptide chain with a molecular weight of 16.5Kd. Several molecular forms of bFGF have been discovered which differ in the length at their amino terminal region. However the biological function of the various

molecular forms appears to be the same.

**[0074]** An embodiment of the invention will now be described by example only and with reference to the following figures:

5 Figure 1: Illustrates expression vector pcDNA3 Tyr-ATA carrying a 570bp insert that includes a tRNA TyrATA gene that is absent from CHO cells;

Figure 2: Illustrates RT-PCR assay demonstrating expression of human tRNA Tyr-ATA gene in stably-transfected CHO cells;

10 Figure 3: Illustrates a transgenic cluster carrying three human tRNA genes with no equivalent in the CHO genome. These were incorporated cloned into pcDNA3 to create pcDNA.YIN, and stably transfected into CHO cells;

15 Figure 4: Illustrates RT-PCR assay demonstrating expression of human tRNA Tyr-ATA, tRNA Asn-ATT and tRNA Ile-GAT genes in CHO cells transfected with pcDNA.YIN;

Figure 5: Illustrates the expression vector pCET1006L&H encoding Herceptin light and heavy chains;

20 Figure 6: Illustrates the effects on relative expression of secreted Herceptin (trastuzumab) of transiently transfecting cloned cells carrying stably incorporated pCET1006L&H with empty vector, pcDNA.YIN + pSUPER.7SL, pcDNA.YIN + vector, or pSUPER-7SL + vector. Mean relative expression (n=3) was vector 1.00, pcDNA.YIN + pSUPER-7SL 1.49, pcDNA.YIN + vector 1.20, pSUPER-7SL + vector 1.24;

25 Figure 7: Illustrates the relative expression of secreted Herceptin (trastuzumab) by matched clones of cells stably transfected with pCET1006L&H with or without pcDNA.YIN. The ratio of secreted Herceptin (trastuzumab) relative to secreted fibronectin (a CHO gene product, control) was increased by -59% with pcDNA.YIN (n=4);

30 Figure 8: Illustrates the percentage of Herceptin (trastuzumab) polypeptides secreted by matched clones of cells stably transfected with pCET1006L&H with or without pcDNA.YIN that have lysine erroneously incorporated instead of asparagine at residue 156 of the light chain and residues 307, 336 and 405 of the heavy chain;

Figure 9: Illustrates vector pRS426, which received a 300bp fragment of *Schizosaccharomyces pombe* genomic sequence containing the tRNA49-LeuCAG gene (SEQ ID NO 9), to create pAU39;

35 Figure 10: Illustrates expression of COG8 and Pgk1, as determined by western blot, in *S. cerevisiae* with or without plasmid pAU39 expressing tRNA-LeuCAG (n=3);

Figure 11: Illustrates vector pESC-TRP, which was used to express in *S. cerevisiae* the turmeric enzyme diketide-CoA synthase (DCS); and

40 Figure 12: Illustrates expression of DCS and Pgk1, as determined by western blot, in *S. cerevisiae* with or without plasmid pAU39 expressing tRNA-LeuCAG.

## Materials and Methods

### 45 Cloning a human tRNA-Tyr-ATA gene into pcDNA3

**[0075]** A 500bp genomic DNA fragment (sequence ID NO 1) containing the human tRNA-Tyr-ATA gene (chr2:219110270-219110770) with the addition of an *attB* sequence at the 5' end and flanked by *Bgl*II (5') and *Xho*I (3') restriction enzyme sequences was synthesised by Life Technologies and provided in the vector pMK-Tyr\_ATA. The sequence was released by digestion with *Bgl*II and *Xho*I at 37°C and gel purified. The eukaryotic expression vector pcDNA3 was digested with *Bgl*II and *Xho*I to remove the CMV promoter, and gel purified. The two DNA fragments were joined using T4 ligase (NEB) and introduced into chemically induced competent *E. coli* by heat shock for 1 minute at 42°C. Plasmid was subsequently isolated from the resulting carbenicillin resistant (50µg/ml) colonies and the presence of the insert confirmed by restriction digest and subsequent sequencing.

55



SEQ ID NO 4:

*Bam*HI / *Xba*I / *Pme*I

AGATCTTCTAGAGTTTAAACCGCCGCTCACAGTACCCGAAAGGCGCATTTCACAAAC  
 TGTGTAGAAAGAGTTCATGGCGGGGTGGCGGCGGGAAAAAAAAAAAAAAAAAGCTAACG  
 CCGTGGCCGGTGCGGGAGTCGAGCCCGCGACCTTGGTGTTATCAGCACCACGCTCTT  
 ACCAACTGAGCTAACCGGCCCGCCTGACGGCACAGTGCCCCTGGAGCGTATAAAAGGG  
 CTCCACGTTTCGCGCCCGTCCACGAGCGATTCTCGCCAAGCCCGCGCGCCTCTGGCT  
 GCCTTCCGCTTTGCCCCAGCCGCGGGGACTCCGGGGAGGAGCCACCGCCCCAGCGC  
 TGGCCGGGTTTCTCCCGCTCCTCCCTGCCAGGCTGTCTCTGACAACCTCTGGGCCGA  
 GTCCAAGAGATCCCGCAAGTCGCTCCTGCCCGCATGGGGCTTACAGAACCTGAAAGGC  
 GCATTTTGAAAAGAGTTGCAGAAACAGTTCATGGAAGCTTAAGCTTCGTTTCAGCTTCC  
 CTTGGGCCCCCTGGCTGCTCGGGCCCGGATCGCGGACCGGGGCGTTTCCGGGGATTT  
 CTGAAGCGGACGAGGGGCAGGGCGGGCGAAGGCCATTTCGGCTCTCCTTCTGGCTCCA  
 GAATCTCCCAACCCGCAGGTGTGCAGTGTGACCAGCGCGACTCACCGCTCTAATCGCT  
 CCGATTTTCCAAGGCCTTGCTCAGTAGTCCTGCCAGGCGGGCTCTGAGGCTGGAAGG  
 GATTGGGGAGTTCGGTGAGTGCGCCCTGCCTATAGCGCCAGTGGAATCGCTAGTACC  
 TGTCTCTGTGGCGCAATCGGTCAGAGCGTTCGGCTATTAACCGAACGGTGAGTAGTT  
 CAAGACCACCCAGGGACGCCTGTTCTAGCTTTT■■■■AAAGCATTTCATGTATTATCATCA  
 CTAGAGAATCTCCCCCTATTCTTCCCATAGTCCTAAGTCCGAAAGGTTGGTTCCAGGCC  
 AGCGGCGCGCCGCTAGCAGATCT

*Asc*I / *Nhe*I / *Bam*HI

#### Introduction of YIN tRNA Gene Cluster into CHO cells and Confirmation of Expression.

**[0080]** pcDNA-YIN was transfected stably into CHO K1 cells, as described above for pcDNA-Tyr-ATA. Expression of each tRNA was confirmed in transfectants (Figure 4), as described above for tRNA-Tyr-ATA. Primer sequences were (SEQ ID NO 5: Forward-TCAGGCGGCCGTTAGC, SEQ ID NO 6: Reverse - GCTAACGCCGTGGCCGGTG) for tRNA-Ile-GAT and (SEQ ID NO 7 Forward - TCGCTAGTACCTGTCTCTGTGG and SEQ ID NO 8: Reverse - CCTGGTGGTCTTGA ACTACTC) for tRNA-Asn-ATT.

#### Creation of Stably-Transfected Clones Expressing Herceptin (Trastuzumab)

**[0081]** One day prior to the transfection,  $3 \times 10^5$  CHO cells were plated in 1 ml of complete growth medium (F12/DMEM) in 6-well Costar clear TC treated cell culture plates (Corning) so that the cells were 50-70% confluent at the time of transfection. In a microcentrifuge tube, 5  $\mu$ g of plasmid pCET1006L+H (Figure 5) DNA encoding Herceptin (trastuzumab) was diluted with Xfect Reaction Buffer to a final volume of 100  $\mu$ l and mixed by vortexing for 5 sec at high speed. 1.5  $\mu$ l Xfect Polymer was added to the diluted plasmid DNA, mixed well by vortexing for 10 sec at high speed (the ratio of Polymer:DNA held constant: 0.3  $\mu$ l of Xfect Polymer per 1  $\mu$ g of plasmid DNA). The mixture was incubated for 10 min at room temperature to allow nanoparticle complexes to form. The entire 100  $\mu$ l of nanoparticle complex solution was added dropwise to the cell culture medium in a well. Plates were incubated at 37°C, 5% CO<sub>2</sub> for 4 hr to overnight, then

nanoparticle complexes were removed from cells by aspiration, replaced with 2-3 ml fresh complete growth medium and the plates were returned to the 37°C incubator. After 24hrs, the cells were trypsinised, counted and resuspended at 200, 500 or 1000 cells/ml in ClonaCell™ semi-solid media (Stem Cell Technologies) supplemented with Glutamax (Gibco) and puromycin (10µg/ml) in untreated plates. The plates were incubated at 37°C, 5% CO<sub>2</sub> for 14-21 days until the colonies were clearly visible. Individual colonies were pipetted off into liquid F12 media, supplemented with 10% FCS and 10ug/ml puromycin, and expanded, before analysis by Western blot.

**[0082]** To assay expression of Herceptin (trastuzumab), 1ml of medium was removed to an Eppendorf tube and spun at 1200rpm to pellet any cellular debris. Supernatant (880µl) was taken off and frozen at -80°C. Samples of medium (30µl) were mixed with 10µl alkylating loading buffer, (125mM Tris, 20% glycerol 2% SDS, 90mM N-Ethylmaleimide (NEM) Bromophenol blue) and incubated at RT for 15 mins. Samples were then heated at 95°C for 10 mins and cooled on ice before SDS-PAGE. Protein was transferred to membrane (Amersham Protran 0.45micron NC # 10600003) pre-soaked in transfer buffer (Towbin-25mM Tris-HCl, 192mM Glycine, pH8.3, 20% MeOH) using a Hoefer Semiphor semi-dry blotter. The membrane was rinsed in 1X PBS and blocked O/N at 4 °C in 5% milk powder, 1% PVP-40 (Sigma 101616021), 0.05% Tween-20 (Applichem GmbH A4974) in 1X PBS). Blocking buffer was then removed and replaced with an HRP conjugated anti-human IgG antibody at a dilution of 1/5000. (Abcam 97165 1mg/ml Goat pAb to human IgG HRP) and incubated for 1 hr at RT with shaking. The membrane was then washed 3x 10 mins in 1x PBS + 0.05% Tween 20. Proteins were detected by adding #34087 Thermo Super-Signal Western Pico Chemi substrate. Excess reagent was removed and signal detected with a SynGene G box chemiimager with GeneSnap software. Membranes were then rinsed in 1X PBS and Fibronectin antibody Ab 2413 (Abcam) added at a 1/10000 dilution in blocking buffer or Sigma F6140 Fibronectin mouse monoclonal 1/4000 dilution blocked in 5% BSA (Sigma A7906) and incubated O/N at 4 °C. Membranes then were washed 3x 10 mins 1x PBS + 0.05% Tween 20, then secondary antibody added in milk blocking buffer (Anti-rabbit IgG HRP 7074S Cell Signalling at a dilution of 1/5000 or anti-mouse HRP 1/5000 dilution #7076P2 Cell Signalling) and incubated 1hr at RT with shaking.

**[0083]** Membrane was washed 3x10 mins in PBS/Tween and developed using high sensitivity substrate as above. Chemiimages were analysed using image J (<https://imagej.nih.gov/ij/index.html>).

#### Effects of Wobble Suppression by YIN on Expression of Herceptin (Trastuzumab)

**[0084]** Clones stably transfected with pCET1006L+H were used to test how expression of Herceptin (trastuzumab) responds to wobble suppression by transient or stable introduction of human tRNA-IleGAT1-1, tRNA-AsnATT1-1 and tRNA-TyrATA genes using pcDNA.YIN. CHO cells do not have these three tRNAs and therefore rely on wobble to decode ATC, AAT and TAT codons for Ile, Asn and Tyr, respectively.

**[0085]** Clones expressing stably transfected pCET1006L+H were grown at 37°C in 5% CO<sub>2</sub> in Hams F12 medium with L-glutamine (Lonza BE12615F), supplemented with 10 % foetal calf serum (Gibco 10270), 100 U/ml Pen/Strep (Gibco 15140122) and 10µg/ml Puromycin (Gibco A11138) and transfected with pcDNA.YIN or equal amounts of pcDNA.3 empty vector, using Genecel. Response to overexpression of 7SL RNA was tested in parallel, by cotransfection of pSUPER-7SL (Misra et al. (2005) J. Biol. Chem 280, 29364), with equal amounts of pcDNA.YIN or empty vector. Levels of Herceptin (trastuzumab) secreted into medium were determined by western blot, as above, 3 days after transfection (Figure 6).

**[0086]** To test the effect of stably expressing tRNA-IleGAT1-1, tRNA-AsnATT1-1 and tRNA-TyrATA, clones stably transfected with pCET1006L+H were further transfected with pcDNA.YIN, as above. After 24hrs, the cells were trypsinised, counted and resuspended at 200, 500 or 1000 cells/ml in ClonaCell™ semi-solid media (Stem Cell Technologies) supplemented with Glutamax (Gibco) and puromycin (10µg/ml) (Gibco A11138), for continued selection of pCET1006L+H integration, and 800µg/ml G418 (Sigma **A1720**) for selection of pcDNA.YIN integration. The plates were incubated at 37°C, 5% CO<sub>2</sub> for 14-21 days until the colonies were clearly visible. Individual colonies were pipetted off into liquid F12 media, supplemented with 10% FCS, 800µg/ml G418 and 10ug/ml puromycin, and expanded. These and parental clones were cultured in T25 cell culture flasks (Starsted) at 37°C in 5% CO<sub>2</sub> in Hams F12 medium with L-glutamine (Lonza BE12615F), supplemented with 10 % foetal calf serum (Gibco 10270), 100 U/ml Pen/Strep (Gibco 15140122) and 10µg/ml Puromycin (Gibco A11138), with or without 800µg/ml G418 (Sigma **A1720**), as appropriate. To assay expression of Herceptin (trastuzumab), 500,000 cells (at passages 4-8) were plated in 5ml of medium in a T25 flask, as above. After 72hrs, a 1ml aliquot of medium was removed to an Eppendorf tube and spun at 1200rpm to pellet any cellular debris. Supernatant (880µl) was taken off and frozen at -80. Expression was assayed subsequently by western blot, as described above (Figure 7).

#### Effects of Wobble Suppression on Misincorporation of Lysine for Asparagine in Herceptin (Trastuzumab)

**[0087]** For analysis by mass spectrometry (MS), Herceptin was purified from cell culture supernatant (F12:DMEM medium with 10% Ultra-Low IgG FBS [Thermo Scientific]) harvested 72-96 hr after plating of single cell clones of stably

expressing CHO cells, using Pierce Protein A Magnetic Beads. (Thermo Scientific Pierce 50 $\mu$ l, 0.5mg) Magnetic Beads were pipetted to a microcentrifuge tube. The tube was placed on the magnet to separate the beads from the storage solution. The Magnetic Beads were washed with 1ml wash buffer (TBS containing 0.05% Tween-20 Detergent), the beads were collected by magnet and the supernatant removed. 30ml of the cell culture supernatant was concentrated to 1.5ml using Amicon Ultra-15 Centrifugal Filter Units with Ultracel-50 membrane (Merck Millipore), added to the beads and incubated with rotation for 60 min at room temperature. The tube was placed on the magnet and the supernatant removed. The beads/antibody complex was resuspended in 500 $\mu$ l wash buffer and washed by gentle pipetting. The beads/antibody complex was washed in total 3 times and separated on the magnet between each wash. The bead suspension was transferred to a clean tube to avoid co-elution of proteins bound to the tube wall. 100 $\mu$ l of elution buffer (50mM glycine pH 2.8) was added to the beads for 10 min to elute the antibody and the buffer was neutralised by adding 15 $\mu$ l 1M TRIS pH 7.5. The eluted protein was mixed with LDS sample buffer (Biorad, 4:1) and reduced by DTT (final concentration 100mM). The mixture was heated for 10 min at 95°C and loaded onto a 4-15% SDS-PAGE gel (Biorad). The gel was run according to the manufacturer's instructions (180V, 30-40 min). The SDS-PAGE gel was stained using InstantBlue Coomassie-based protein stain (Expedeon). In-gel tryptic digestion was performed after reduction with DTE and S-carbamidomethylation with iodoacetamide. Gel pieces were washed twice with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in 50 mM acetic acid, then diluted 5-fold with 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02  $\mu$ g/ $\mu$ L. Gel pieces were rehydrated by adding 25  $\mu$ L of trypsin solution, and after 10 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37°C. Peptides were extracted by washing three times with 50% (v:v) aqueous acetonitrile containing 0.1% trifluoroacetic acid (v:v), before drying in a vacuum concentrator and reconstituting in aqueous 0.1% trifluoroacetic acid (v:v). Peptides were loaded onto an UltiMate 3000 RSLCnano HPLC system (Thermo) equipped with a PepMap 100 Å C<sub>18</sub>, 5  $\mu$ m trap column (300  $\mu$ m  $\times$  5 mm, Thermo) and an Acclaim PepMap RSLC or EasyNano column, 2  $\mu$ m, 100 Å, (C<sub>18</sub>, 75  $\mu$ m  $\times$  150 mm, Thermo). The trap wash solvent was aqueous 0.05% (v:v) trifluoroacetic acid and the trapping flow rate was 15  $\mu$ l/min. The trap was washed for 3 min before switching flow to the capillary column. The separation used a gradient elution of two solvents (solvent A: aqueous 1% (v:v) formic acid; solvent B: aqueous 80% (v:v) acetonitrile containing 1% (v:v) formic acid). The flow rate for the capillary column was 300 nl/min. Column temperature was 40°C and the gradient profile was: linear 3-10% B over 7 mins, linear 10-35% B over 30 mins, linear 35-99% B over 5 mins then proceeded to wash with 99% solvent B for 4 min. The column was returned to initial conditions and re-equilibrated for 15 min before subsequent injections.

**[0088]** The nanoLC system was interfaced with an Orbitrap Fusion hybrid mass spectrometer (Thermo) with a Nano-spray Flex or EasyNano ionisation source (Thermo). Positive ESI-MS and MS<sup>2</sup> spectra were acquired using Xcalibur software (version 4.0, Thermo). Instrument source settings were: ion spray voltage, 1,900-2,400 V; sweep gas, 2 Arb; ion transfer tube temperature; 275°C. MS<sup>1</sup> spectra were acquired in the Orbitrap with common acquisition parameters among all acquisitions, specifically: 120,000 resolution; scan range, m/z 375-1,500; AGC target, 4e<sup>5</sup>; max fill time, 100 ms and data type, profile. MS<sup>2</sup> spectra were acquired in top speed mode maintaining a fixed cycle time of 1 s; quadrupole isolation window, m/z 1.6; activation type, HCD; collision energy, 32%; first mass, m/z 110 and data type, centroid. To maximise peptide identifications, multiple MS<sup>2</sup> acquisition regimes were performed using both the linear ion trap and Orbitrap mass analysers. Linear ion trap MS<sup>2</sup> acquisitions specified scan rate, rapid; AGC target, 5e<sup>3</sup> and max injection time, 100 ms. Orbitrap MS<sup>2</sup> acquisitions used resolution, 30,000; AGC target, 5e<sup>4</sup> and max injection time, 54 ms. Data dependent precursor selection was selected either the most or least (separate acquisitions) intense precursor above a threshold of 5e<sup>3</sup>. Dynamic exclusion was applied for 50 s post precursor selection and a minimum threshold for fragmentation was set at 5e<sup>3</sup>. Latterly, a targeted approach was taken for MS<sup>2</sup> acquisitions using a fixed inclusion list containing the theoretical 2+ and 3+ m/z values for peptides previously identified as containing Asn to Lys modification.

**[0089]** Peak lists were generated in MGF format using MSConvert (proteowizard, version 3.0.9967). MGF files were searched against the expected protein sequences, appended to an in-house database, using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.5.1). Search criteria specified: Enzyme, semi-trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M), Asn -> Lys (N); Peptide tolerance, 3 ppm; MS/MS tolerance, 0.5 Da for linear ion trap or 10 mDa for Orbitrap; Instrument, ESI-TRAP or ESI-ORBITRAP-HCD. Peptides classified as containing amino acid substitutions were required to have expect scores <0.05 and were manually validated to ensure the presence of sequence ions localising the position of substitution to the suggested amino acid.

**[0090]** Extracted ion chromatogram peak areas were generated using Qual Browser (Xcalibur, version 4.0, Thermo), setting a 2 mDa window around expected precursor m/z values. XICs were Gaussian smoothed and peak picking parameters specified: baseline window, 100; area noise factor, 5; peak noise factor, 100. Percentage of amino acid substitution was estimated by comparing the peptide XIC areas of the substituted form with the sum of the substituted and expected forms.

**[0091]** Figure 8 shows the frequency of lysine misincorporation in matched clones with or without pcDNA.YIN. Data are given for four sites where misincorporation of lysine was detected consistently. In each case, the correct residue,

asparagine, is encoded by an AAT codon that depends on wobble in native CHO cells, but can be decoded without wobble by tRNA-AsnATT in cells transfected with pcDNA.YIN.

### Cloning a fission yeast tRNA-LeuCAG gene into pRS426

**[0092]** A 300bp fragment (sequence ID NO 9) of genomic DNA from chromosome I of the fission yeast *Schizosaccharomyces pombe* containing the tRNA<sup>49</sup>-LeuCAG gene (chr1:1527350-1527050) flanked by *Bam*HI and *Spe*I restriction enzyme sites at the upstream and downstream ends, respectively, was synthesised by Life Technologies. The sequence was released by digestion with *Bam*HI and *Spe*I at 37°C and gel purified. The DNA fragment was introduced into vector pRS426 (Figure 9) cut with *Bam*HI- and *Spe*I, to create plasmid pAU39, using T4 ligase (NEB) at room temperature for 2hrs, and introduced into chemically-induced competent *E. coli* (*DH5α*) by heat shock for 0.5 minutes at 42°C. Plasmid pAU39 was subsequently isolated from the resulting ampicillin-resistant (100μg/ml) colonies and the presence of the insert confirmed by restriction digest and subsequent sequencing.

SEQ ID NO 9:

*Bam*HI

GGATCCTTTTCGTTCAATCAAGATATTTTATCACATTATTTCAAGGTTCAATGAATCATT  
 GAAGAATAGTAACGAATTCAGATATATTAATAAAGGCACACATAATCTCTAAAGATTAC  
 AGTTTAATGGCTCTTTCAAAGTGTTTAGTAATATATACACTAAATTTCTAAAATTCAGTAG  
 TTATGGCGAAGTGGCCGAGTGGTCTATGGCGCTAGCTTCAGGTGAAAGCAATTGCTAG  
 TCTACGTATGTGGGCGTGGGTTCGAACCCACCTTCGTCAATATAATTTTTTATATCATG  
 TTAATGGAATTGAGAAAACTAGT

*Spe*I

### Effect of fission yeast tRNA-LeuCAG on transgene expression in budding yeast

**[0093]** *Saccharomyces cerevisiae* strain COG8-TAP (genotype *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*, COG8-TAP::HIS3, Mat a) was transformed using lithium acetate with pAU39 or empty pRS426 vector. Transformants were grown in standard minimal media (SD, lacking amino acids where appropriate for plasmid selection). 5 OD<sub>600</sub> unit equivalents of cells were harvested from liquid cultures in log phase and resuspended in 120μl lysis buffer prior to incubation at 95°C for 2 min and vortexing for 3min in the presence of 100μg glass beads (425-600 μm, acid washed). Lysates were resolved by 8% SDS PAGE and then analysed by western blotting with antibodies against COG8 and loading control Pgk1, as above (Figure 10).

**[0094]** Strain G175 (*ADE2 MET his3 leu2 ura3 trp1* TAG+ SE+, Mat α) was also transformed with pAU39 or empty pRS426, along with vector pESC-TRP (Figure 11) with or without a transgene containing the coding sequence of diketide-CoA synthase (DCS; AB495006.1), taken from turmeric (*Curcuma longa*), attached to a Myc tag. This sequence contains 17 CTG codons that depend on wobble for expression in native *S. cerevisiae*, but not after introduction of the exogenous tRNA-LeuCAG gene on pAU39. Expression of DCS was measured by western blotting, as above, with antibody against the Myc tag (Figure 12).

### Example 1

**[0095]** CHO cells do not have genes for tRNA-IleGAT, tRNA-AsnATT or tRNA-TyrATA and therefore rely on wobble to decode TAT, AAT and ATC codons. To allow wobble-independent decoding of these codons, we made and introduced a construct, pcDNA.YIN, carrying human tRNA-IleGAT, tRNA-AsnATT and tRNA-TyrATA genes. A DNA fragment containing the tRNA<sup>14</sup>-TyrATA gene from human chromosome 2 was synthesized and cloned (Fig 1). The construct was stably transfected into CHO cells and RT-PCR analysis confirmed expression of the transgene (Fig. 2). The same approach was used to introduce a synthetic assembly (Fig 3), in pcDNA.YIN, expressing human tRNA-TyrATA, tRNA-AsnATT and tRNA-IleGAT. Expression of all three human tRNAs in recipient CHO cells was confirmed by RT-PCR (Fig.

4). The effect of these tRNAs was tested on expression of Herceptin (trastuzumab) in CHO cells stably transfected with pCET1006L+H expression vector (Fig. 5). These cells were transfected with empty vector, pcDNA.YIN and/or pSUPER-7SL and secreted Herceptin (trastuzumab) was harvested and quantified (Fig. 6). Transient transfection of pcDNA.YIN was found to raise Herceptin expression by -20%. This enhancement was boosted to -49% by cotransfection of pSUPER-7SL, a construct containing the human RN7SL1 gene, that encodes 7SL RNA. In stable transfectants, genomic incorporation of pcDNA.YIN was found to raise by -59% expression of Herceptin (trastuzumab), relative to the parental clone without pcDNA.YIN (Figure 7). Mass spectrometry was used to ascertain rates of lysine misincorporation in the secreted antibody due to misreading of the wobble-dependent AAT codon for asparagine. Such errors were detected at 4 positions, with frequencies up to -0.28%. These errors were suppressed by 4- to 32-fold in matched cells carrying the pcDNA.YIN, where the exogenous tRNA<sup>Asn</sup>-ATT allows direct decoding of the AAT codon without wobble (Figure 8). Overall, these experiments with a therapeutic antibody demonstrate increased production and decreased misincorporation following introduction into CHO cells of exogenous tRNAs that reduce dependence on wobble.

## Example 2

**[0096]** *S. cerevisiae* relies on wobble to decode the CTG codon for leucine, as it has no tRNA-LeuCAG. To remove the wobble-dependence of this codon, we created a strain of *S. cerevisiae* that carries an exogenous tRNA-LeuCAG gene from *Schizosaccharomyces pombe*. The effect of this tRNA was measured on expression of genes that are rich in CTG codons. A DNA fragment containing the trna49-LeuCAG gene from chromosome 1 of *S. pombe* was synthesized and cloned into pRS426 (Figure 9) to create plasmid pAU39. Western blotting was used to determine the effect of pAU39 on expression in *S. cerevisiae* of COG8 (Figure 10) and DCS (Figure 12). There are 10 CTG codons in COG8 and 17 in DCS, but none in P<sub>gk1</sub>, an endogenous protein that was used as control. For both COG8 and DCS, expression was enhanced by the tRNA-LeuCAG gene on pAU39 relative to the empty vector control. We conclude that the efficacy of *S. cerevisiae* as a platform for expressing transgenes can be improved by introduction of exogenous tRNA to reduce dependence on wobble.

## Claims

1. An isolated eukaryotic cell, wherein said isolated eukaryotic cell is not a human germ cell, **characterized in that** said cell is modified by transfection or transformation with a nucleic acid molecule comprising a transcription cassette, the transcription cassette comprising at least one nucleotide sequence encoding a transfer RNA [tRNA],

the tRNA including an anticodon nucleotide sequence selected from the group consisting of AGC, GGC, CGC, UGC, ACC, GCC, CCC, UCC, AGG, GGG, CGG, UGG, AGU, GGU, CGU, UGU, AAC, GAC, CAC, UAC, AAA, GAA, AUU, GUU, CUU, UUU, AUC, GUC, CUC, UUC, AGA, GGA, CGA, UGA, ACU, GCU, ACG, GCG, CCG, UCG, CCU, UCU, AAG, GAG, CAG, UAG, CAA, UAA, AAU, GAU, UAU, CAU, AUA, GUA, ACA, GCA, AUG, GUG, CUG, UUG, CCA and

that is absent from said eukaryotic cell, wherein said anticodon sequence corrects base mismatches at any one of the cognate first, second or third nucleotide position in the corresponding messenger RNA [mRNA] codon to improve translation efficiency and/or decrease amino acid mis-incorporation as a consequence of degeneracy in the genetic code, and wherein said base mismatch correction reduces said cells reliance on wobble during translation of said mRNA.

2. The isolated eukaryotic cell according to claim 1, wherein said nucleic acid molecule is part of an expression vector adapted for eukaryotic expression of said tRNA.

3. The isolated eukaryotic cell according to any one of claims 1 to 2 wherein said cell is further modified by transformation or transfection with a nucleic acid molecule encoding a recombinant protein, polypeptide or peptide.

4. The isolated eukaryotic cell according to claim 3 wherein said modified cell is transformed or transfected with a nucleic acid molecule encoding a therapeutic antibody, a pharmaceutical or therapeutic protein or peptide or an industrial enzyme.

5. The isolated eukaryotic cell according to claim 3 wherein said modified cell is transformed or transfected with a nucleic acid molecule encoding therapeutic antibody.

6. The isolated eukaryotic cell according to claim 5 wherein said therapeutic antibody is a chimeric or humanised

therapeutic antibody.

7. The isolated eukaryotic cell according to any one of claims 1 to 6 wherein said isolated eukaryotic cell is an insect, plant, fungal or mammalian cell.

8. The isolated eukaryotic cell according to claim 7 wherein said isolated eukaryotic cell is a mammalian cell.

9. The isolated eukaryotic cell according to claim 8 wherein said mammalian cell is a Chinese Hamster Ovary cell, HEK293, NS0 or CAP cells.

10. The isolated eukaryotic cell according to any one of claims 1 to 9 wherein said nucleic acid encodes a transfer RNA selected from the group of Ala-tRNA, Arg-tRNA, Asp-tRNA, Asn-tRNA, Cys-tRNA, Glu-tRNA, Gln-tRNA, Gly-tRNA, His-tRNA, Ile-tRNA, Leu-tRNA, Lys-tRNA, Met-tRNA, Phe-tRNA, Pro-tRNA, Ser-tRNA, Thr-tRNA, Trp-tRNA, Tyr-tRNA and Val-tRNA.

11. The isolated eukaryotic cell according to any one of claims 1 to 10 wherein said modified cell is further modified by transformation or transfection with a nucleic acid molecule comprising a 7SL nucleotide sequence.

12. A method for reducing tRNA mismatch as a consequence of the degeneracy in the genetic code comprising the steps:

- i) providing a modified cell according to any one of claims 1 to 11;
- ii) incubating the cell in i) under cell culture conditions suitable for expression of one or more recombinant proteins/peptides expressed by said modified cell; and optionally
- iii) isolating the expressed recombinant proteins/peptides from the modified cell or cell culture medium.

13. A method to customise expression of a selected recombinant protein in a cell expression system comprising the steps:

- i) analysing the nucleotide sequence of a nucleic acid molecule encoding a recombinant protein to be expressed to identify one or more codon/anticodon tRNA mismatches;
- ii) transfecting or transforming a cell with one or more transcription cassettes comprising one or more tRNA genes that correct at least one base pairing mismatch in at least one codon to provide a modified cell;
- iii) transfecting or transforming said modified cell with the recombinant protein to be expressed; and optionally
- iv) providing cell culture conditions to express said recombinant protein.

14. A cell culture vessel comprising a modified cell according to any one of claims 1 to 11.

## Patentansprüche

1. Isolierte eukaryontische Zelle, wobei die isolierte eukaryontische Zelle keine menschliche Keimzelle ist, **dadurch gekennzeichnet, dass** die Zelle durch Transfektion oder Transformation mit einem Nukleinsäuremolekül modifiziert ist, das eine Transkriptionskassette umfasst, wobei die Transkriptionskassette mindestens eine Nukleotidsequenz umfasst, die eine [tRNA] codiert,

wobei die tRNA eine Anticodon-Nukleotidsequenz beinhaltet, ausgewählt aus der Gruppe bestehend aus AGC, GGC, CGC, UGC, ACC, GCC, CCC, UCC, AGG, GGG, CGG, UGG, AGU, GGU, CGU, UGU, AAC, GAC, CAC, UAC, AAA, GAA, AUU, GUU, CUU, UUU, AUC, GUC, CUC, UUC, AGA, GGA, CGA, UGA, ACU, GCU, ACG, GCG, CCG, UCG, CCU, UCU, AAG, GAG, CAG, UAG, CAA, UAA, AAU, GAU, UAU, CAU, AUA, GUA, ACA, GCA, AUG, GUG, CUG, UUG, CCA, und das in der eukaryontischen Zelle fehlt, wobei die Anticodonsequenz Basenfehlpaarungen an einer beliebigen Position des verwandten ersten, zweiten oder dritten Nukleotids in dem entsprechenden Messenger-RNA-[mRNA-]Codon korrigiert, um die Translationseffizienz zu verbessern und/oder die fehlerhafte Integrierung von Aminosäuren als Folge einer Degeneration des genetischen Codes zu verringern, und wobei die Basenfehlpaarungskorrektur die Abhängigkeit der Zellen von Wobble während der Translation der mRNA reduziert.

2. Isolierte eukaryontische Zelle nach Anspruch 1, wobei das Nukleinsäuremolekül Teil eines Expressionsvektors ist, der für die eukaryontische Expression der tRNA angepasst ist.

3. Isolierte eukaryontische Zelle nach einem der Ansprüche 1 bis 2, wobei die Zelle durch Transformation oder Transfektion mit einem Nukleinsäuremolekül, das ein rekombinantes Protein, Polypeptid oder Peptid codiert, weiter mo-

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difiziert ist.

4. Isolierte eukaryontische Zelle nach Anspruch 3, wobei die modifizierte Zelle mit einem Nukleinsäuremolekül, das einen therapeutischen Antikörper, ein pharmazeutisches oder therapeutisches Protein oder Peptid oder ein industrielles Enzym codiert, transformiert oder transfiziert ist.
5. Isolierte eukaryontische Zelle nach Anspruch 3, wobei die modifizierte Zelle mit einem Nukleinsäuremolekül, das einen therapeutischen Antikörper codiert, transformiert oder transfiziert ist.
6. Isolierte eukaryontische Zelle nach Anspruch 5, wobei der therapeutische Antikörper ein chimärer oder humanisierter therapeutischer Antikörper ist.
7. Isolierte eukaryontische Zelle nach einem der Ansprüche 1 bis 6, wobei die isolierte eukaryontische Zelle eine Insekten-, Pflanzen-, Pilz- oder Säugetierzelle ist.
8. Isolierte eukaryontische Zelle nach Anspruch 7, wobei die isolierte eukaryontische Zelle eine Säugetierzelle ist.
9. Isolierte eukaryontische Zelle nach Anspruch 8, wobei die Säugetierzelle eine Eierstockzelle des chinesischen Hamsters, HEK293, NS0- oder CAP-Zelle ist.
10. Isolierte eukaryontische Zelle nach einem der Ansprüche 1 bis 9, wobei die Nukleinsäure eine Transfer-RNA codiert, ausgewählt aus der Gruppe bestehend aus Ala-tRNA, Arg-tRNA, Asp-tRNA, Asn-tRNA, Cys-tRNA, Glu-tRNA, Gln-tRNA, Gly-tRNA, His-tRNA, Ile-tRNA, Leu-tRNA, Lys-tRNA, Met-tRNA, Phe-tRNA, Pro-tRNA, Ser-tRNA, Thr-tRNA, Trp-tRNA, Tyr-tRNA und Val-tRNA.
11. Isolierte eukaryontische Zelle nach einem der Ansprüche 1 bis 10, wobei die modifizierte Zelle durch Transformation oder Transfektion mit einem Nukleinsäuremolekül, das eine 7SL-Nukleotidsequenz umfasst, weiter modifiziert ist.
12. Verfahren zum Reduzieren von tRNA-Fehlpaarung als Folge der Degeneration im genetischen Code, umfassend die folgenden Schritte:
  - i) Bereitstellen einer modifizierten Zelle nach einem der Ansprüche 1 bis 11;
  - ii) Inkubieren der Zelle aus i) unter Zellkulturbedingungen, die für die Expression eines oder mehrerer rekombinanter Proteine/Peptide geeignet sind, die von der modifizierten Zelle exprimiert werden; und optional
  - iii) Isolieren der exprimierten rekombinanten Proteine/Peptide aus der modifizierten Zelle oder dem Zellkulturmedium.
13. Verfahren zum individuellen Anpassen der Expression eines ausgewählten rekombinanten Proteins in einem Zell-expressionssystem, umfassend die folgenden Schritte:
  - i) Analysieren der Nukleotidsequenz eines Nukleinsäuremoleküls, das ein zu exprimierendes rekombinantes Protein codiert, um eine oder mehrere Codon/Anticodon-tRNA-Fehlpaarungen zu identifizieren;
  - ii) Transfizieren oder Transformieren einer Zelle mit einer oder mehreren Transkriptionskassetten, die ein oder mehrere tRNA-Gene umfassen, die mindestens eine Fehlpaarung der Basenpaarung in mindestens einem Codon korrigieren, um eine modifizierte Zelle bereitzustellen;
  - iii) Transfizieren oder Transformieren der modifizierten Zelle mit dem zu exprimierenden rekombinanten Protein; und optional
  - iv) Bereitstellen von Zellkulturbedingungen zum Exprimieren des rekombinanten Proteins.
14. Zellkulturgefäß, umfassend eine modifizierte Zelle nach einem der Ansprüche 1 bis 11.

### Revendications

1. Cellule eucaryote isolée, ladite cellule eucaryote isolée n'étant pas une cellule germinale humaine, **caractérisée en ce que** ladite cellule est modifiée par transfection ou transformation avec une molécule d'acide nucléique comprenant une cassette de transcription, la cassette de transcription comprenant au moins une séquence nucléotidique codant un ARN de transfert [ARNt],

- l'ARNt comprenant une séquence nucléotidique d'anticodon choisie dans le groupe constituée par AGC, GGC, CGC, UGC, ACC, GCC, CCC, UCC, AGG, GGG, CGG, UGG, AGU, GGU, CGU, UGU, AAC, GAC, CAC, UAC, AAA, GAA, AUU, GUU, CUU, UUU, AUC, GUC, CUC, UUC, AGA, GGA, CGA, UGA, ACU, GCU, ACG, GCG, CCG, UCG, CCU, UCU, AAG, GAG, CAG, UAG, CAA, UAA, AAU, GAU, UAU, CAU, AUA, GUA, ACA, GCA, AUG, GUG, CUG, UUG, CCA et qui est absente de ladite cellule eucaryote, ladite séquence d'anticodon corrigeant les mésappariements de bases au niveau de l'une quelconque parmi la première, la deuxième ou la troisième position de nucléotide analogue dans le codon d'ARN messenger [ARNm] correspondant pour améliorer l'efficacité de traduction et/ou diminuer la mésincorporation d'acides aminés à la suite de la dégénérescence du code génétique, et ladite correction de mésappariements de bases réduisant ladite dépendance des cellules à l'égard de l'oscillation pendant la traduction dudit ARNm.
2. Cellule eucaryote isolée selon la revendication 1, ladite molécule d'acide nucléique faisant partie d'un vecteur d'expression adapté à l'expression eucaryote dudit ARNt.
  3. Cellule eucaryote isolée selon l'une quelconque des revendications 1 à 2, ladite cellule étant en outre modifiée par transformation ou transfection avec une molécule d'acide nucléique codant une protéine recombinante ou un polypeptide ou peptide recombinant.
  4. Cellule eucaryote isolée selon la revendication 3, ladite cellule modifiée étant transformée ou transfectée avec une molécule d'acide nucléique codant un anticorps thérapeutique, une protéine ou un peptide pharmaceutique ou thérapeutique, ou une enzyme industrielle.
  5. Cellule eucaryote isolée selon la revendication 3, ladite cellule modifiée étant transformée ou transfectée avec une molécule d'acide nucléique codant un anticorps thérapeutique.
  6. Cellule eucaryote isolée selon la revendication 5, ledit anticorps thérapeutique étant un anticorps thérapeutique chimérique ou humanisé.
  7. Cellule eucaryote isolée selon l'une quelconque des revendications 1 à 6, ladite cellule eucaryote isolée étant une cellule d'insecte, de plante, de champignon ou de mammifère.
  8. Cellule eucaryote isolée selon la revendication 7, ladite cellule eucaryote isolée étant une cellule de mammifère.
  9. Cellule eucaryote isolée selon la revendication 8, ladite cellule de mammifère étant une cellule d'ovaire de hamster chinois, des cellules HEK293, NS0 ou CAP.
  10. Cellule eucaryote isolée selon l'une quelconque des revendications 1 à 9, ledit acide nucléique codant un ARN de transfert choisi dans le groupe de Ala-ARNt, Arg-ARNt, Asp-ARNt, Asn-ARNt, Cys-ARNt, Glu-ARNt, Gln-ARNt, Gly-ARNt, His-ARNt, Ile-ARNt, Leu-ARNt, Lys-ARNt, Met-ARNt, Phe-ARNt, Pro-ARNt, Ser-ARNt, Thr-ARNt, Trp-ARNt, Tyr-ARNt et Val-ARNt.
  11. Cellule eucaryote isolée selon l'une quelconque des revendications 1 à 10, ladite cellule modifiée étant en outre modifiée par transformation ou transfection avec une molécule d'acide nucléique comprenant une séquence de nucléotide 7SL.
  12. Méthode permettant de réduire un mésappariement d'ARNt à la suite de la dégénérescence du code génétique comprenant les étapes de :
    - i) fourniture d'une cellule modifiée selon l'une quelconque des revendications 1 à 11 ;
    - ii) incubation de la cellule en i) dans des conditions de culture cellulaire appropriées à l'expression d'une ou plusieurs protéines/d'un ou plusieurs peptides exprimés par ladite cellule modifiée ; et éventuellement
    - iii) isolement des protéines recombinantes/peptides recombinants exprimés de la cellule modifiée ou du milieu de culture cellulaire.
  13. Méthode permettant d'adapter l'expression d'une protéine recombinante choisie dans un système d'expression cellulaire comprenant les étapes de :
    - i) analyse de la séquence nucléotidique d'une molécule d'acide nucléique codant une protéine recombinante

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destinée à être exprimée pour identifier un ou plusieurs mésappariements d'ARNt de codon/anticodon ;  
ii) transfection ou transformation d'une cellule avec une ou plusieurs cassettes de transcription comprenant un ou plusieurs gènes d'ARNt qui corrigent au moins un mésappariement de paire de bases dans au moins un codon pour fournir une cellule modifiée ;  
5 iii) transfection ou transformation de ladite cellule modifiée avec la protéine recombinante destinée à être exprimée ; et éventuellement  
iv) fourniture de conditions de culture cellulaire pour exprimer ladite protéine recombinante.

14. Cuve de culture cellulaire comprenant une cellule modifiée selon l'une quelconque des revendications 1 à 11.

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Figure 1

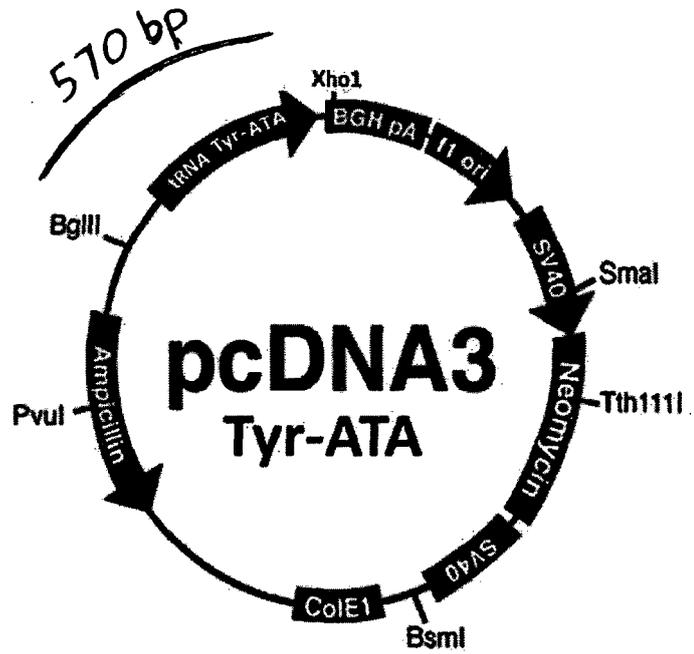


Figure 2

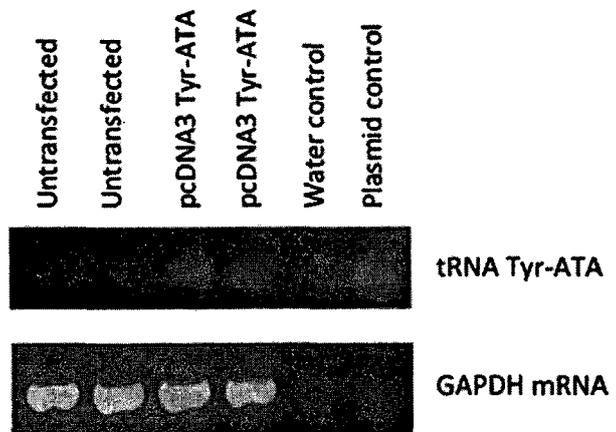
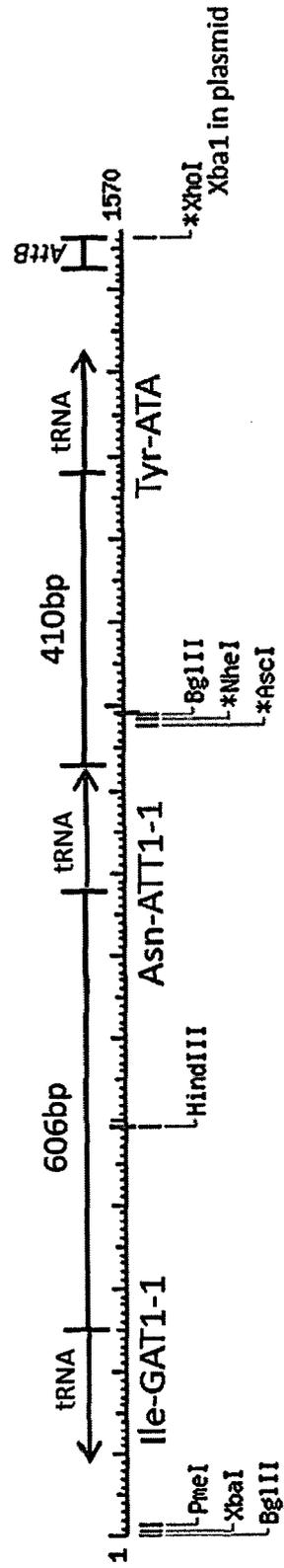


Figure 3

### Human tRNA assembly



Cloned into promoterless pcDNA3

Figure 4

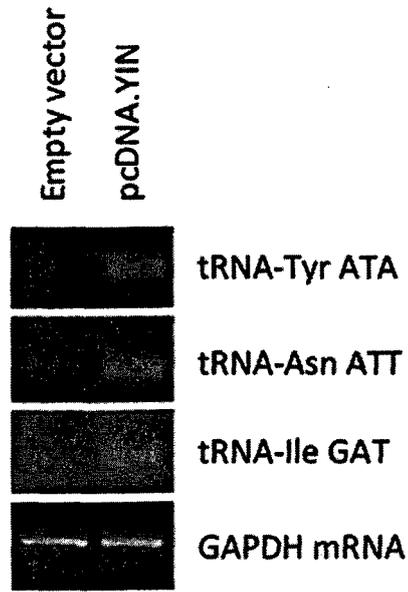


Figure 5

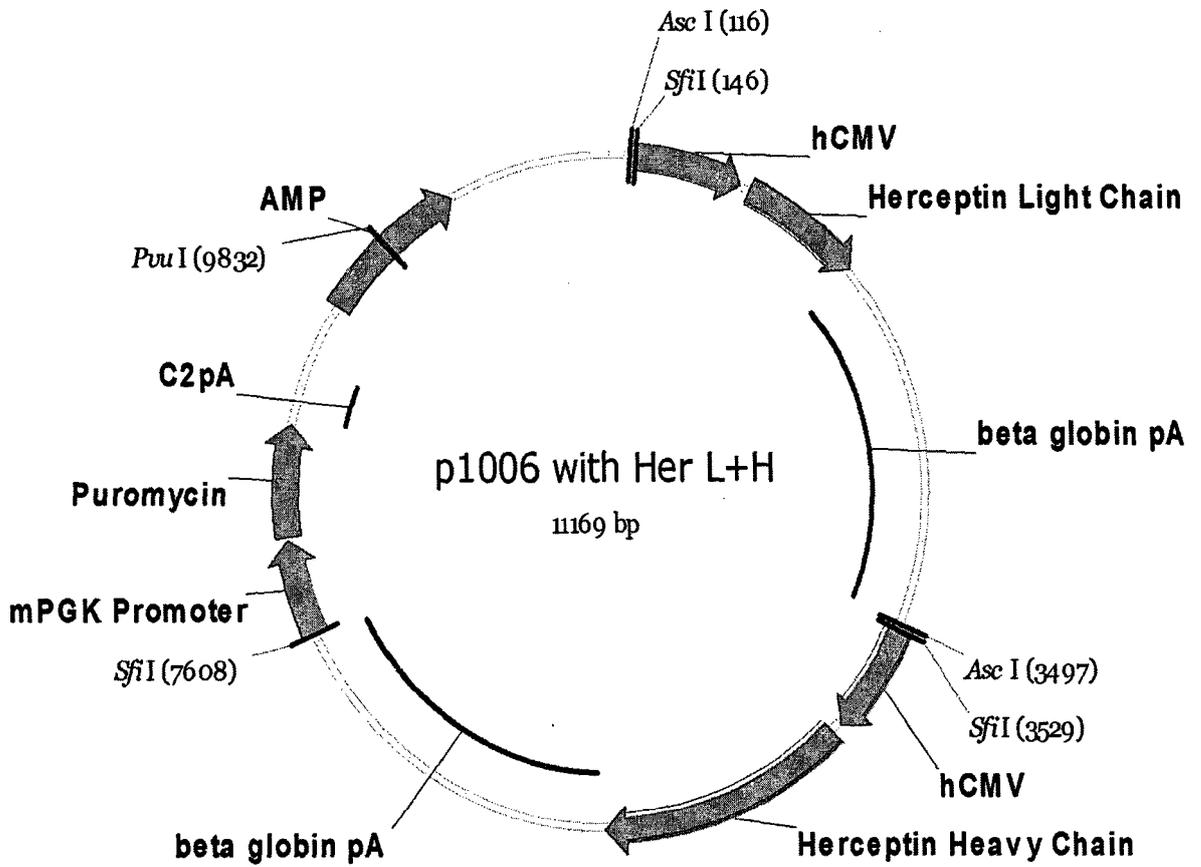


Figure 6

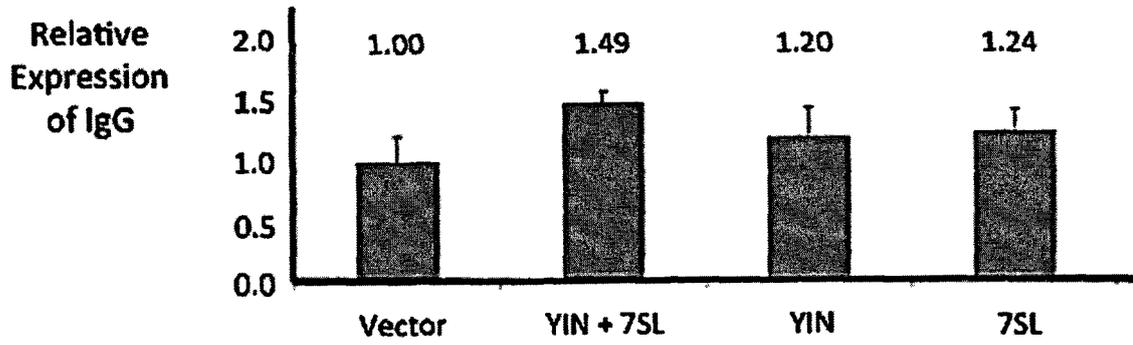
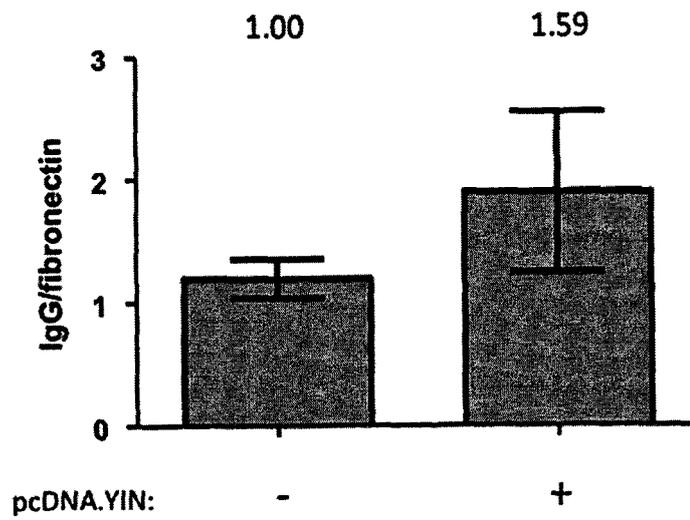


Figure 7



**Figure 8**

Site of Error	Error Rate -YIN	Error Rate +YIN	Error Suppression
L chain N156	0.0466%	0.00147%	32-fold
H chain N307	0.0102%	0.00114%	9-fold
H chain N336	0.277%	0.0263%	11-fold
H chain N405	0.147%	0.037%	4-fold

Figure 9

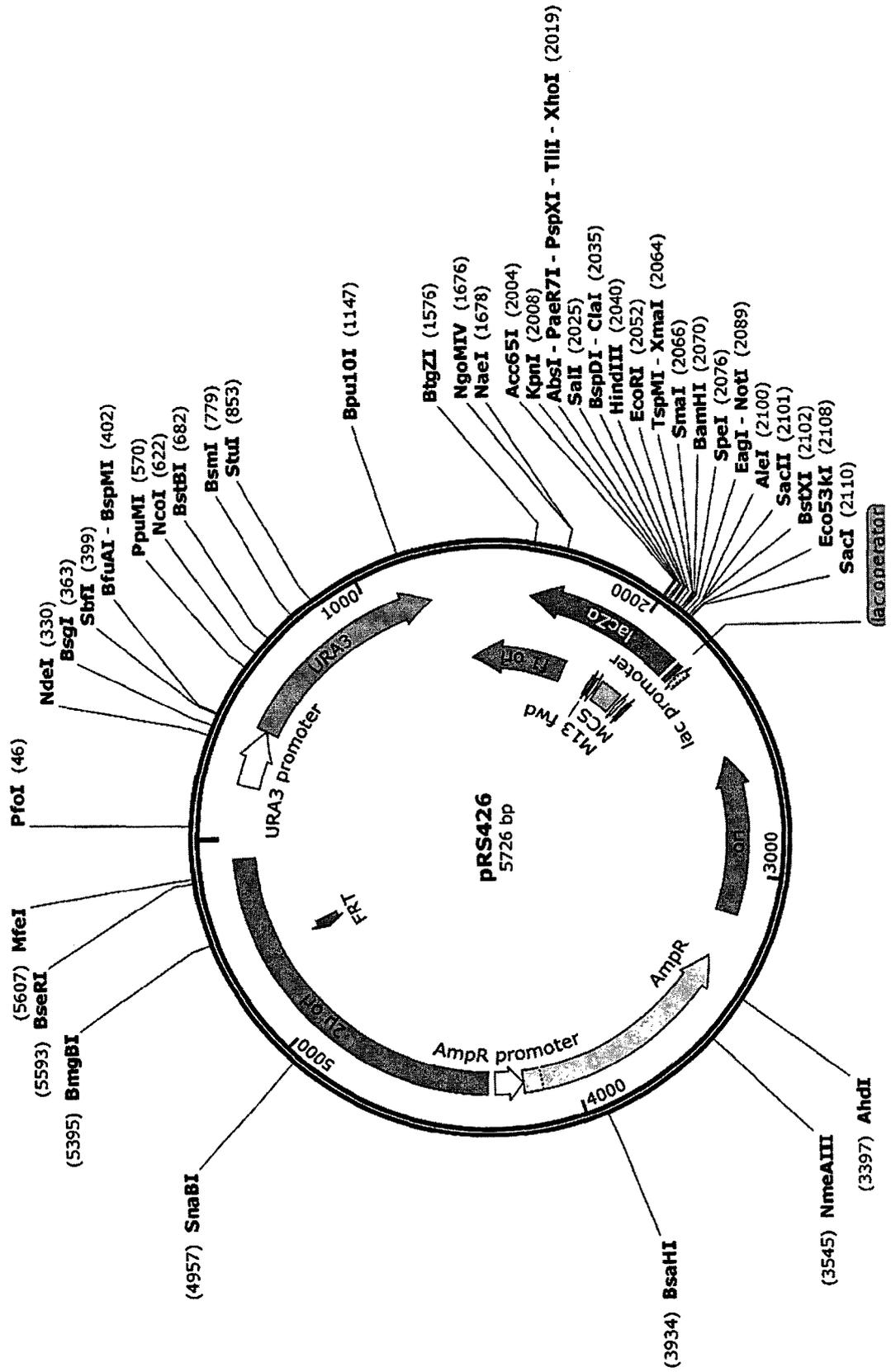


Figure 10

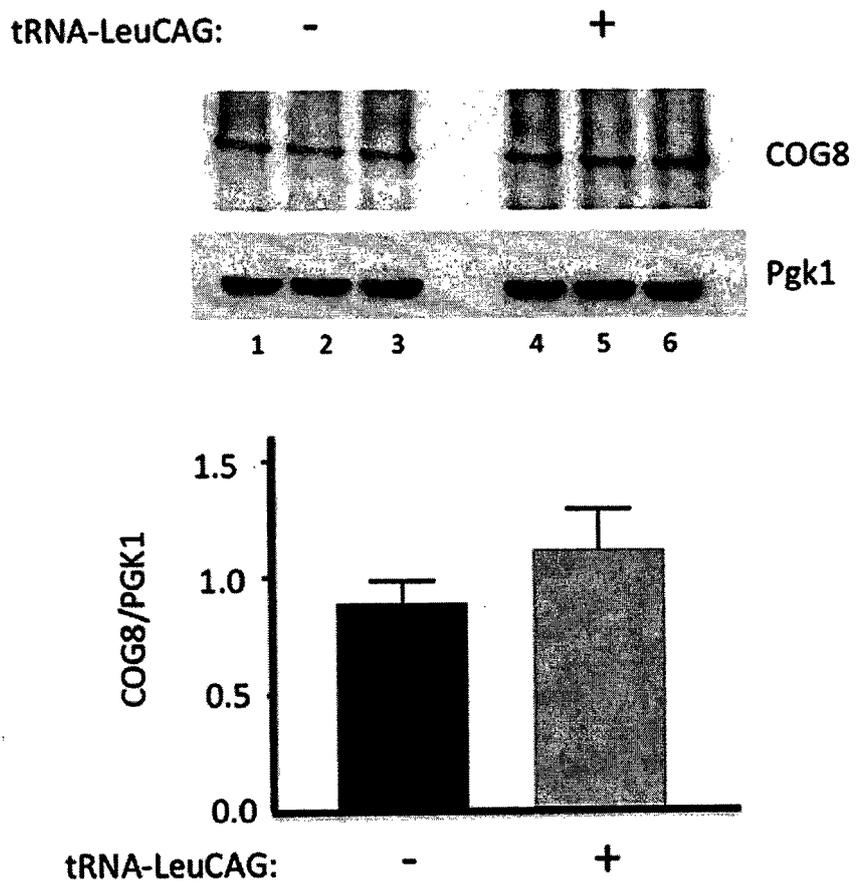


Figure 11

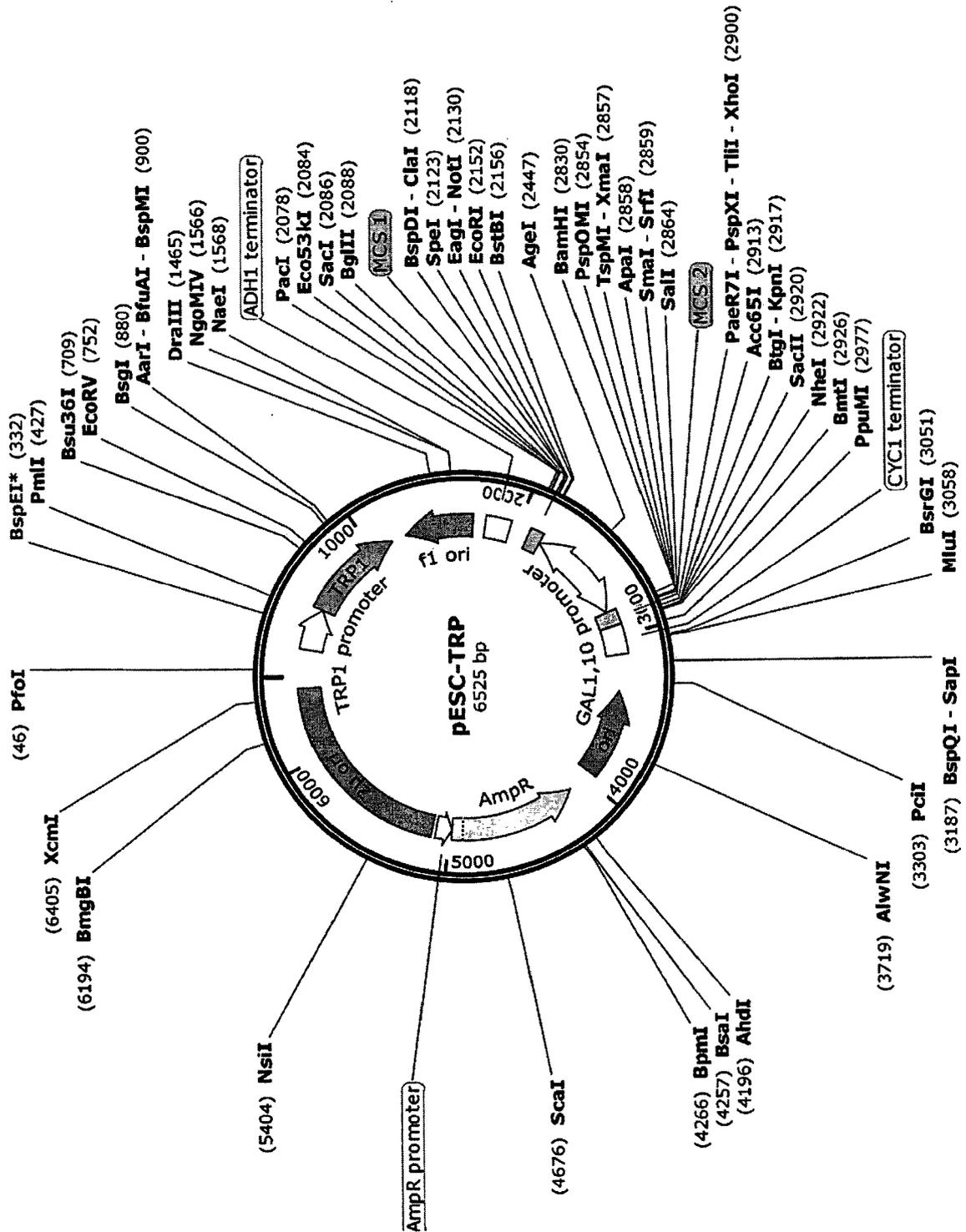
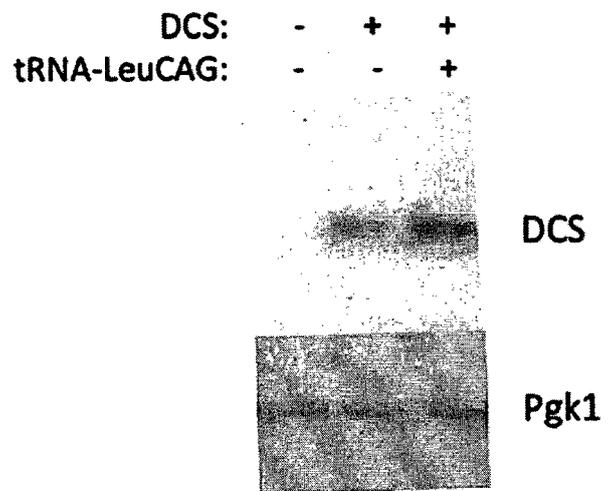


Figure 12



## REFERENCES CITED IN THE DESCRIPTION

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## Patent documents cited in the description

- WO 2007103307 A [0006]
- WO 2004085463 A [0006]
- US 2005287639 A [0006]
- WO 2014160032 A [0006]
- EP 1911840 A [0006]
- EP 2246428 A [0006]
- WO 2004024915 A [0006]
- US 2013149699 A [0006]
- WO 2015048989 A [0006]
- WO 9807876 A [0014]
- US 409297 [0020]
- US 5608149 A [0020]
- US 5608144 A [0020]
- US 5604121 A [0020]
- US 5569597 A [0020]
- US 5466785 A [0020]
- US 5399680 A [0020]
- US 5268463 A [0020]
- US 5608142 A [0020]
- US 5814618 A [0020]
- US 5789156 A [0020]
- EP 194809 A [0022]
- US 6248516 B [0069]

## Non-patent literature cited in the description

- JOHANSSON et al. *PNAS*, 2012, vol. 109 (1), 131-136 [0006]
- IBEN et al. *RNA*, 2012, vol. 18 (7), 1358-1372 [0006]
- HERVE SELIGMANN. *Computational Biology and Chemistry*, 2011, vol. 35 (2), 81-95 [0006]
- WALD et al. *Nucleic Acids Research*, 2012, vol. 40 (15) [0006]
- GROSVELD et al. *Cell*, 1987, vol. 51, 975-985 [0015]
- SAMBROOK et al. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour Laboratory, 1989 [0015]
- MARSTON, F. *DNA Cloning Techniques: A Practical Approach*. IRL Press, 1987, vol. III [0015]
- F M AUSUBEL et al. *Current Protocols in Molecular Biology*. John Wiley & Sons, Inc, 1994 [0015]
- FENG et al. *Nature Biotechnology*, 1997, vol. 15, 866-870 [0016]
- ODELL et al. *Nature*, 1985, vol. 313, 9810-812 [0020]
- MCELROY et al. *Plant Cell*, 1990, vol. 2, 163-171 [0020]
- CHRISTIAN et al. *Plant Mol. Biol.*, 1989, vol. 18, 675-689 [0020]
- LAST et al. *Theor Appl. Genet.*, 1991, vol. 81, 581-588 [0020]
- VELTEN et al. *EMBO J.*, 1984, vol. 3, 2723-2730 [0020]
- SCHENA et al. *Proc. Natl. Acad. Sci. USA*, 1991, vol. 88, 10421-10425 [0020]
- MCNELLIS et al. *Plant J*, 1998, vol. 14 (2), 247-257 [0020]
- GATZ et al. *Mol. Gen. Genet.*, 1991, vol. 227, 229-237 [0020]
- YAMAMOTO et al. *Plant J*, 1997, vol. 12 (2), 255-265 [0021]
- KAWAMATA et al. *Plant Cell Physiol.*, 1997, vol. 38 (7), 792-803 [0021]
- HANSEN et al. *Mol. Gen. Genet.*, 1997, vol. 254 (3), 337-343 [0021]
- RUSSELL et al. *Transgenic Res.*, 1997, vol. 6 (2), 157-168 [0021]
- RINEHART et al. *Plant Physiol.*, 1996, vol. 112 (3), 1331-1341 [0021]
- VAN CAMP et al. *Plant Physiol.*, 1996, vol. 112 (2), 525-535 [0021]
- CANEVASCNI et al. *Plant Physiol.*, 1996, vol. 112 (2), 513-524 [0021]
- YAMAMOTO et al. *Plant Cell Physiol.*, 1994, vol. 35 (5), 773-778 [0021]
- LAM. *Results Probl. Cell Differ.*, 1994, vol. 20, 181-196 [0021]
- OROZCO et al. *Plant Mol. Biol.*, 1993, vol. 23 (6), 1129-1138 [0021]
- MUTSUOKA et al. *Proc. Natl. Acad. Sci. USA*, 1993, vol. 90 (20), 9586-9590 [0021]
- GUEVARA-GARCIA et al. *Plant J.*, 1993, vol. 4 (3), 495-50 [0021]
- Plant transformation and expression vectors. GUERINEAU ; MULLINEAUX. *Plant Molecular Biology Labfax*. BIOS Scientific Publishers, 1993, 121-148 [0022]
- Manual of Methods for General Bacteriology. American Society for Bacteriology, 1981 [0058]
- PAUL. *Fundamental Immunology* [0069]
- JANEWAY et al. *Immunobiology* [0069]

- MISRA et al. *J. Biol. Chem.*, 2005, vol. 280, 29364  
[0085]