

Novel biologicals production system

Field

- 5 The application relates to an expression system for the expression of a gene of interest.

Background

Most biopharmaceuticals are produced in higher eukaryotic (commonly mammalian) cell
10 lines because this provides “human-like” post-translational modifications required for
functionality [1]. A cell factory is a genetically modified cell, optimised for the industrial
production of biopharmaceuticals. Historically several human and murine cell lines have
been used as cell factories to produce biopharmaceuticals. Industry standard cell factory
platforms for the manufacture of therapeutic proteins now use variants of Chinese Hamster
15 Ovary (CHO) cells, adapted to industrial bioprocessing methods [2]. Commercial CHO cell
variants are natural or engineered auxotroph's for key nutrients essential for cell growth such
as amino acids or nucleosides [3]. This may be leveraged to provide a selection system.
There are two key commercial selection systems, which rely on the enzymes Glutamine
synthase (GS) and Dihydrofolate reductase (DHFR). Both systems are augmented by small
20 molecular weight inhibitors of the enzymes: methionine sulfoximine (MSX) for GS and
methotrexate (MTX) for DHFR which enable isolation or amplification of cells expressing
the gene of interest which, being genetically linked to the co-expressed recombinant GOI,
leads to higher protein expression making these cells suitable for commercial use.

25 NAD^+ , NADH , NADP^+ and NADPH are crucial for cell function and cell growth, through
coupled dehydrogenase reactions which support catabolic and biosynthetic reactions. These
reactions generate the co-factors required to support cell division and associated metabolic
interconversions. NAD^+ biosynthesis is critical for the biosynthesis of the three other
nicotinamide adenine coenzymes. In mammalian cells NAD^+ synthesis occurs via one of
30 three routes: Preiss-Handler; *de-novo* and a salvage pathway (which is shown in Figure 1).
The salvage pathway is key to maintenance of cellular pools of NAD^+ [4].

Biosynthesis of NAD^+ and related coenzymes is accomplished either through a *de
novo* pathway from tryptophan or from a synthesis pathway utilising nicotinamide as a

synthesis precursor. The *de novo* pathway enzymes are predominantly localised in liver cells in mammals [4]. Nicotinamide phosphoribosylation via the synthesis pathway represents the major route for NAD⁺ synthesis in Chinese Hamster Ovary (CHO) cells. Phosphoribosylation of nicotinic acid acts as a minor alternative precursor for synthesis using a specific phosphoribosyl transferases to shared final interconversions in the *de novo* synthetic pathway [5, 6, 7].

Over the past 5-10 years there has been increasing attention on the significance of NAMPT in the development of tumour cells where high expression of NAMPT has been associated with malignant phenotype [8].

Several inhibitors of NAMPT (e.g. GMX1778 and FK866) have been developed [9], which exhibit clinical promise in several tumour cell therapies [10, 11]. The importance of NAMPT in cellular NAD⁺ synthesis has been reinforced by the identification of compounds which selectively activate NAMPT in cultured human lung cells, resulting in increased cellular NAD⁺ concentrations [12]. These studies demonstrate the critical importance of the synthesis pathway of NAD⁺ biosynthesis for cell growth and health, which with specific, potent inhibitors of NAMPT identifies a highly tractable system to enhance cell metabolism and biosynthesis.

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Summary

In a first aspect of the invention, an expression system for selecting the presence of a gene of interest is provided, the expression system comprising:

- 25 (i) a cell line wherein the activity of at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited,
the cell line optionally further comprising the gene of interest, and the cell line optionally lacking an endogenous nucleotide sequence which encodes the at least one enzyme in the NAD⁺ synthesis pathway;
- 30 (ii) an expression vector comprising an exogenous nucleotide sequence which encodes the at least one enzyme in the NAD⁺ synthesis pathway; and
- (iii) a media formulation which does not comprise nicotinamide (NAM) and/or nicotinic acid.

In another aspect of the invention, a cell line wherein the activity of at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited, optionally further comprising a gene of interest, and optionally lacking an endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway is provided.

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In another aspect of the invention, an expression vector comprising an exogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway and optionally a gene of interest is provided.

- 10 In another aspect of the invention, a media formulation which does not comprise nicotinamide (NAM) and/or nicotinic acid is provided.

In some embodiments, the gene of interest is encoded by the expression vector and/or by a second vector.

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In some embodiments, the cell line comprises the expression vector.

In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is selected from nicotinamide phosphoribosyltransferase (NAMPT), nicotinic acid
20 phosphoribosyltransferase (NPRT) and/or quinolinic acid phosphoryltransferase (QPRT).

In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is a heterologous mammalian NAMPT.

- 25 In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway has at least 70% sequence identity to SEQ ID 1 to 8.

In some embodiments, the gene of interest encodes a recombinant protein, viral vector, component of a viral vector, viral vaccine, intracellular protein and/or receptor.

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In some embodiments, the exogenous nucleotide sequence encoding the at least an enzyme in the NAD⁺ synthesis pathway is under the control of a promoter, optionally wherein the promoter is CMV, SV40 and/or mPGK.

In some embodiments, the gene of interest is under the control of a promoter, optionally wherein the promoter is CMV, SV40 and/or mPGK.

In some embodiments, the cell line is a eukaryotic cell line, optionally, selected from:
5 Chinese hamster ovary K1 cells; HEK cells; and/or VERO cells.

In some embodiments, the cell line is genetically engineered to lack the endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway.

10 In some embodiments, the media formulation further comprises an inhibitor, optionally wherein the inhibitor is a transferase inhibitor.

In some embodiments, the transferase inhibitor is a Nicotinamide Phosphoribosyl Transferase (NAMPT) Inhibitor.

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In some embodiments, the media formulation is a substantially serum-free and/or protein-free medium.

In another aspect of the invention, a method for selecting cells comprising a gene of interest
20 using the expression system, cell line, expression vector or media formulation is provided.

In another aspect of the invention, a method of culturing and/or expanding the cell line using the media formulation is provided.

25 In another aspect of the invention, a use of the expression system is provided, wherein cells that do not comprise at least one enzyme in the NAD⁺ synthesis pathway do not survive.

Brief description of the drawings

30 Embodiments of the invention will now be described, by way of example only, with reference to accompanying drawings, in which:

Figure 1 shows the synthesis pathway as a route for NAD⁺ synthesis.

Figure 2 is a schematic diagram on an exemplary embodiment of the inventive expression system.

Figure 3 is a schematic overview of components of the expression system.

Figure 4 shows that a transferase inhibitor of NAMPT (FK866) kills cells, and this is prevented with the addition of nicotinic acid.

Figure 5 shows that a transferase inhibitor of NAMPT kills cells, and this is prevented with the addition of nicotinic acid.

Figure 6A and Figure 6B show that cells which do not comprise endogenous NAMPT (sg1-3) cannot survive in the absence of nicotinic acid.

Figure 7A and Figure 7B shows that cells which do not comprise endogenous NAMPT cannot survive in the absence of nicotinic acid.

Figure 8 shows an exemplary embodiment of the expression vector.

Figure 9 shows a western blot of IgG expression from the NAMPT vector shown in Figure 10 in transient and stable transfection.

Figure 10 shows an exemplary embodiment of the expression vector.

Figure 11 shows an exemplary embodiment of the mechanism of the invention.

Figure 12 shows protein expression levels from a knockout transfected with a gene of interest under NAMPT selection.

Figure 13 shows another exemplary embodiment of the mechanism of the invention including gene amplification with the NAMPT inhibitor FK866.

Figure 14A to D show that the addition of the inhibitor FK866 applies selection pressure to result in amplification of protein expression.

Detailed description

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In a first aspect of the invention, an expression system for selecting the presence of a gene of interest is provided, the expression system comprising:

- (i) a cell line wherein the activity of at least one enzyme in the NAD^+ synthesis pathway is at least partially inhibited, optionally lacking an endogenous nucleotide sequence which encodes at least one enzyme in the NAD^+ synthesis pathway, the cell line optionally further comprising the gene of interest;
- (ii) an expression vector comprising an exogenous nucleotide sequence which encodes the at least one enzyme in the NAD^+ synthesis pathway; and

(iii) a media formulation which does not comprise nicotinamide (NAM) and/or nicotinic acid.

5 In another aspect of the invention, a cell line wherein the activity of the at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited, optionally lacking an endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway is provided, optionally the cell line further comprising a gene of interest.

10 In another aspect of the invention, an expression vector comprising an exogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway and optionally a gene of interest is provided.

15 In some embodiments the exogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway may be the same as the endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway. In some embodiments the exogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway may be different to the endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway.

20 In another aspect of the invention, a media formulation which does not comprise nicotinamide (NAM) and/or nicotinic acid is provided.

25 An expression system is a system designed for the production of a gene of interest (GOI). The expression system of the present invention is adapted for and enables selective expression of the GOI. The activity of the at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited, and this results in a reduced or completely eliminated production of NAD⁺. The term "inhibition" as used herein means the reduction or prevention of activity.

30 The activity of the at least one enzyme in the NAD⁺ synthesis pathway may be inhibited by at most about 5, 10, 25, 50, 75, 80, 90, 95, 98, 99, or 100 % compared to the activity before inhibition.

The activity of the at least one enzyme in the NAD⁺ synthesis pathway may be inhibited by at least about 5, 10, 25, 50, 75, 80, 90, 95, 98, 99, or 100 % compared to the activity before inhibition. In some embodiments the activity of the at least one enzyme in the NAD⁺ synthesis pathway may be substantially completely inhibited.

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The inhibitor may reduce cell growth, survival and/or viability of the cell line, prior to the provision of expression vector comprising an exogenous nucleotide sequence which encodes the at least one enzyme in the NAD⁺ synthesis pathway. The inhibitor may reduce cell growth, survival and/or viability by at most about 5, 10, 25, 50, 75, 80, 90, 95, 98, 99, or 100 %.

10 The inhibitor may reduce cell growth, survival and/or viability by at least about 5, 10, 25, 50, 75, 80, 90, 95, 98, 99, or 100 %. This may be measured using optical density (OD), VCD or any other measurements as known in the art.

15 In some embodiments, the method of inhibition is selected from small molecule or via gene expression.

In some embodiments, the inhibition of the endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway is via inactivation or substantially inactivating the enzyme.

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In embodiments wherein the method of inhibition is via gene expression, the cell line may lack an endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway. Thus, the cell line does not substantially express at least one enzyme in the NAD⁺ synthesis pathway and cannot substantially make NAD⁺. The cell line may be

25 known as an auxotrophic cell line. In some embodiments, the cell line lacks an endogenous nucleotide sequence encoding one enzyme in the NAD⁺ synthesis pathway.

In embodiments wherein the method of inhibition is via gene expression, the inhibitor may be small interfering RNAs (siRNA). In such embodiments, the expression of the at least one

30 enzyme in the NAD⁺ synthesis pathway is reduced by RNA interference (RNAi). The system of siRNA delivery may be non-viral or viral delivery systems. The non-viral delivery system may include polymers, optionally comprising lipid and/or peptide complexes. Said lipid and/or peptide complexes may facilitate passage of siRNA into appropriate cellular compartments to initiate RNA interference reactions.

In some embodiments, the cell line is a natural variant, which lacks or has substantially low expression of the endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway.

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In some embodiments, the endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway is inhibited. In some embodiments, the cell line is engineered to switch off, knock out or otherwise remove or substantially inactivate the endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway. This improves expression and stability of gene expression of the recombinant gene during culture scale-up (prolonged cell culture).

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In some embodiments, the cell line is genetically engineered to lack the endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway. In some embodiments, endogenous nucleotide sequence is deleted or removed to generate the cell line. This provides the advantage of more stringent selection. In some embodiments, a knockout system is used to generate the cell line, which may include adenovirus, zinc fingers, mega nuclease, TALENs and/or other mechanisms known in the art. In some embodiments, CRISPR, for example CRISPR-Cas9 or Cas-Clover, is used to generate the cell line. This provides the advantage that the gene deletion is rapid.

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In a specific example, CRISPR-Cas9 is used to knockout the NAMPT gene in the cells. This makes use of optimised delivery conditions of the CRISPR components (such as single guide RNA (sgRNA) and Cas9 protein) to the specific CHO cell variant using control guide RNA (gRNA) known to target the genome, for example lipofection or nucleofection. Three to four sgRNA targeted against NAMPT may be synthesised, including low off targeting potential, targeted against asymmetric exons, and against exons common to all isoforms. sgRNA may be co-delivered with Cas9 according to the established protocols, and efficiency of editing measured using sequencing and TIDE analysis. Groups of cells with the highest level of editing may be taken forward for monoclonal derivation. Clones may be screened both genetically (to determine the InDels created in the gene, and confirm clonality) and proteomically (via western blot) to confirm loss of NAMPT (high quality commercial antibodies are available against NAMPT).

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In another example, and adeno-associated virus (AAV) may be used to remove the endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway.

- 5 Cell lines that are deficient in or inactivated an endogenous nucleotide sequence encoding at least a first enzyme in the NAD⁺ synthesis pathway would be severely metabolically impaired, and so would be unlikely or unable to survive in nicotinamide-deficient medium but will grow in medium supplemented with nicotinic acid. Cells transfected with the vector would be selected through combined use of nicotinamide- and nicotinic acid-deficient
10 culture medium.

The inventors used RNAseq studies on CHO K1 cells and CHO-S cells to show that there is no expression of mRNA encoding for indole 2,3-dioxygenase, kynureninase or quinolinate phosphoribosyltransferase. This data verify that industrially relevant CHO cells do not
15 express components of the de novo synthetic pathway that enables tryptophan catabolism into precursors for NAD synthesis. In contrast, mRNA encoding enzymes needed to convert nicotinic acid (NA) or nicotinamide (NAM) to NAD are all expressed although NARPT (that will handle NA) is expressed at only 12-15% of NAMPT. This shows that NAMPT is a powerful selection marker.

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In embodiments wherein the method of inhibition is small molecule, the inhibitor is a small molecular weight inhibitor of the at least one enzyme in the NAD⁺ synthesis pathway.

In some embodiments, the inhibitor is a transferase inhibitor. In some embodiments, the
25 inhibitor is a Nicotinamide Phosphoribosyl transferase (NAMPT) Inhibitor. The Nicotinamide Phosphoribosyl transferase (NAMPT) Inhibitor may selected from: (E)-Daporinad (FK866); CHS-828 (GMX1778); OT-82; Padnarsertib (KPT-9274); GNE-618, STF-118804; GNE-617 hydrochloride; Teglarinad chloride (GMX1777); GPP78 (CAY10618); Nampt-IN-3 (Compound 35); Nampt-IN-5; CB30865 (ZM 242421); LB-60-
30 OF61, CB 300919; NAMPT inhibitor-linker 2; LB-60-OF61 hydrochloride; Nampt-IN-9 (Compound 8); NAMPT/IDO1-IN-1; or a combination thereof. The transferase inhibitor may be selected for its efficacy and/or specificity. Some transferase inhibitors are known for providing additional activities, for example may be associated with anti-cancer activities.

Preferably, the transferase inhibitor is FK866 or GMX1778. These inhibitors enjoy the advantage that their activity profiles are known and understood. In addition, these inhibitors are capable of producing up to complete inhibition of cell growth at low concentrations.

- 5 The inhibitor enables isolation or amplification of cells with higher gene copies which, being linked to the co-expressed recombinant GOI, leads to higher protein expression making these cells suitable for commercial use. Thus, the inhibitor augments the selection of the cell line.

Such inhibitors allow enhanced selection of highly expressing clones containing multiple
10 gene copies, and are likely to select for cells with additional capacity to synthesize NAD^+ . This is advantageous for cell health and protein production.

In some embodiments, an exogenous nucleotide sequence which encodes the at least one enzyme in the NAD^+ salvage pathway is provided. This restores the NAD^+ salvage pathway,
15 and enables the cell line to survive. Figure 2 shows an exemplary embodiment of this.

Advantageously, the expression of the exogenous nucleotide sequence which encodes the at least one enzyme in the NAD^+ salvage pathway will allow expression of the GOI, and so only cells which express the at least one enzyme in the NAD^+ salvage pathway and the GOI
20 can survive. Co-expression of the at least one enzyme in the NAD^+ salvage pathway restores the ability to synthesise the key nutrient, allowing selection of only those cells expressing the GOI. Cells which take up the expression vector are selected using the media formulation that is deficient in the key nutrient.

- 25 The expression system enjoys the advantage of generating highly stringent metabolic selection but with significant advantages over the known systems, such as the GS system. NAD(P)^+ is an essential cofactor widely involved in cellular functions, multiple metabolic pathways and the maintenance and repair of DNA. GS is much less widely involved in cellular functions and can be substituted with other amino acids such as asparagine and
30 glutamate, thus reducing the stringency of GS selection. The claimed expression system utilises the NAD^+ salvage pathway, and therefore is more selective.

In some embodiments, the at least one enzyme in the NAD^+ synthesis pathway is selected from: nicotinamide phosphoribosyltransferase (NAMPT); nicotinic acid

phosphoribosyltransferase (NPRT); quinolinic acid phosphoryltransferase (QPRT); Indoleamine 2,3-dioxygenase 1 and/or 2 (IDOs); Tryptophan 2,3-dioxygenase (TDO); Nicotinamide/nicotinic acid mononucleotide adenylyltransferase 1, 2, and/or 3 (NMNATs); and/or other enzymes involved in the conversion of small molecular weight metabolites and amino acids into precursors of nicotinamide. In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is nicotinamide phosphoribosyltransferase (NAMPT), nicotinic acid phosphoribosyltransferase (NPRT) and/or quinolinic acid phosphoryltransferase (QPRT). These provide the advantage of playing a critical role in the NAD⁺ synthesis pathway and thus providing a more stringent selection.

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NAMPT is a target for novel anticancer drugs, and the inventors expect that overexpression of NAMPT leading to increased metabolism of nicotinamide into the NAD pool, would support rapid cell growth and division creating multiple generations of daughter cells in culture, making this enzyme an ideal metabolic selection marker. In addition, the inventors have shown that nicotinamide is rapidly consumed via the synthesis pathway and is linked to rapid cell growth, in particular in CHO cells.

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In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is two, three or more enzymes. This may improve the selectivity of the expression system. In one specific example, cells may be engineered to regulate the rate of growth may be regulated by controlling the activity of two, three or more enzymes in by in the NAD⁺ synthesis pathway. A network of enzymes, some of which are illustrated in Figure 1, are involved in NAD⁺ synthesis. This network may be engineered to control the NAD⁺ synthesis.

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In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is one enzyme. This provides the advantage of tight control of the system.

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In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway is a heterologous mammalian NAMPT. An enzyme from a mammalian origin may be expressed in a superior manner in mammalian cells. For example, appropriate post-translational modifications may be made, and the expression is optimised for mammalian cells. In some embodiments, the mammalian NAMPT is a human NAMPT, for example Gene ID: 10135 which may be found on the NCBI online National Library of Medicine.

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In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway has at least 70% sequence identity to any one of SEQ ID 1-8. SEQ ID 1-8 represent exemplary sequences of the at least one enzyme in the NAD⁺ synthesis pathway, relating to a human (*Homo sapiens*). However, the person skilled in the art may be able to determine other suitable sequences, and the nucleotide sequences that encode them. In some embodiments, the at least one enzyme in the NAD⁺ synthesis pathway has at least 70% sequence identity to any one of SEQ ID 1 and/or 2.

Within this specification, the term “identity” is used to refer to the similarity of two sequences. For the purpose of this invention, it is defined here that in order to determine the percent identity of two sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first sequence for optimal alignment with a second amino or nucleic acid sequence). The nucleotide/amino acid residues at each position are then compared. When a position in the first sequence is occupied by the same amino acid or nucleotide residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100). Generally, the two sequences are the same length. A sequence comparison is typically carried out over the entire length of the two sequences being compared.

The skilled person will be aware of the fact that several different computer programs are available to determine the identity between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two nucleic acid sequences is determined using the sequence alignment software Clone Manager 9 (Sci-Ed software - www.scied.com) using global DNA alignment; parameters: both strands; scoring matrix: linear (mismatch 2, OpenGap 4, ExtGap 1).

Alternatively, the percent identity between two amino acid or nucleic acid sequences can be determined using the Needleman and Wunsch (1970) algorithm which has been incorporated into the GAP program in the Accelrys GCG software package (available at

http://www.accelrys.com/products/gcg/), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. A further method to assess the percent identity between two amino acid or nucleic acid sequences can be to use the BLAST sequence comparison tool available on the National
5 Center for Biotechnology Information (NCBI) website (www.blast.ncbi.nlm.nih.gov), for example using BLASTn for nucleotide sequences or BLASTp for amino acid sequences using the default parameters.

The expression system, cell line, expression vector and media formulation enjoy the
10 advantage of providing more stringent selection over cell health. They enable a rapid and specific isolation of the desired cells and, by the nature of the metabolic selection, enhance cell growth resulting in selection of greater numbers of recombinant clones, higher biomass in culture and improved overall cell performance as a platform for production of biopharmaceuticals in particular. The selection on the basis of the at least one enzyme in the
15 NAD^+ synthesis pathway means that the system also is compatible with a wide range of mammalian cell types, including CHO cells.

In some embodiments, the gene of interest is encoded by the expression vector and/or by a second vector. In some embodiments, the expression vector comprises both the exogenous
20 nucleotide sequence which encodes the at least one enzyme in the NAD^+ synthesis pathway and the GOI. This provides the advantage of selecting for cells that have successfully been transformed with the expression vector, and thus will be capable of expressing the GOI.

In some embodiments, the cell line comprises both the expression vector and the second
25 vector. For example, the cell line may be co-transformed with the expression vector and the second vector. In this embodiment, the cells which have been successfully transformed with the expression vector will survive and will also comprise the second vector. This provides the advantage that the cell line may be provided in a kit comprising the cell line, media formulation and the expression vector. The user may co-transform the cells with the
30 expression vector and a second vector comprising the GOI, thereby using the expression system to select from cells successfully co-transformed with the expression vector and second vector.

In some embodiments of the invention, the vector may be inserted into the cell line using any suitable means. The means for insertion of the vector into cells may be selected from the group of plasmid transformation (including transfection), viral transduction, or non-viral transfection. Examples of non-viral transfection include use of materials such as polymers, lipids, peptides, inorganic materials and/or hybrid materials. Viral transduction may use viral vectors, such as an Adeno Associated Virus (AAV), Herpes Simplex Virus (HSV), adenovirus, retrovirus, lentivirus, or baculovirus. The vector may be a lentivirus vector or an AAV vector, although in some embodiments, the vector may be any suitable vector for allowing the expression of the at least one second enzyme in the NAD⁺ synthesis pathway and the GOI.

The transfection may be transient or stable. Transient transfection refers to DNA remaining in the nucleus as opposed to integrated into the genome. Transient transfection provides the advantage that it is useful to test if a gene of interest within an expression system is expressed. Stable transfection refers to the DNA being integrated into the genome. Stable transfection provides the advantage that the cell line may be stored and revived as needed. In addition, due to genetic variance, some cells may exhibit improved expression, and these may be selected and reused for industrial uses such as manufacture.

The gene of interest (GOI) refers to a desired nucleotide sequence. The GOI may encode a recombinant protein, viral vector, components of viral vector, viral vaccine, intracellular protein and/or receptor, a viral vector, and/or a receptor.

The recombinant protein may be a secreted protein, for example proteases, glycosidases. The recombinant protein may be a cell-surface protein, for example: MHC proteins; markers of cell lineages, such as IGR, TCRs. The recombinant protein may be a serum protein.

The recombinant protein may be a therapeutic protein or a protein for therapeutic use. The recombinant protein may be a single protein, at least one protein, a protein-based complex. The recombinant protein may be an antibody. The recombinant protein may be selected from: antibodies; antibodies containing non-natural amino acids, optionally for manufacture of antibody-drug conjugates; multi-specific antibodies; domain engineered proteins such as domain-engineered antibodies (such as bi, tri- and multi-specific formats, and/or fusion combinations including protein-Fc structures); and/or monoclonal antibodies (mAbs). In

some embodiments, the antibody is Trastuzumab, also known as Herceptin®. In some embodiments, the recombinant protein may be selected from fluorescent secretory proteins: sEGFP; sRFP; smCherry;

- 5 The intracellular protein may be a protein involved in redirection of cellular functions and regulation.

The viral vector may be selected from: Adeno Associated Virus (AAV); Herpes Simplex Virus (HSV); adenovirus; retrovirus; lentivirus; and/or baculovirus.

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The expression vector and/or selection vector may be deoxyribonucleic acid (DNA). In a specific example, the expression vector is according to Figure 8.

- 15 In some embodiments, the gene of interest and/or the exogenous nucleotide sequence encoding the at least an enzyme in the NAD^+ synthesis pathway is under the control of a promoter, which optionally may be a polymerase type 2 promotor.

- 20 In some embodiments, the promoter is selected from the group including human cytomegalovirus intermediate early promoter (hCMV), mouse cytomegalovirus intermediate early promoter (mCMV), human elongation factor 1 alpha promoter (hEF1 α), mouse elongation factor 1 alpha promoter (mEF1 α), chicken beta actin hybrid promoter (CAG), mouse phosphoglycerate kinase promoter (mPGK), or human ubiquitin C promoter (UBC), a liver specific promoter (including the transthyretin (TTR) promoter and alpha-1-antitrypsin promoter (hAAT)), a ubiquitous promoter (including CAG, CMV, CBh and SV40), a CBh promoter, a transthyretin (TTR) promoter or an alpha-1-antitrypsin promoter (hAAT). In some embodiments, the promoter is a neuron-specific enolase (NSE) promoter. In some embodiments, the promoter is a neuron-specific enolase (NSE) promoter selected from the group synapsin I (SYN) promoter, calcium/calmodulin-dependent protein kinase II (CaMKII) promoter, tubulin alpha I promoter, neuron-specific enolase promoter or platelet-derived growth factor beta chain promoter. In some embodiments, the promoter is a synthetic promotor. In some embodiments the promotor is a modified promotor selected from any of those listed herein.
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In some embodiments, the gene of interest is under the control of a CMV, SV40 and/or mPGK promoter. In some embodiments, the exogenous nucleotide sequence encoding the at least an enzyme in the NAD⁺ synthesis pathway is under the control of a mPGK promoter. Thus, the expression vector and/or the second vector may comprise at least one promoter. In
5 embodiments in which the gene of interest and the at least an enzyme in the NAD⁺ synthesis pathway are encoded in the expression vector, the expression vector may comprise two promoters. SV40, CMV AND mPGK provide the advantage of being suitable for expression control.

10 The promotor may be selected to improve expression. The promotor may be selected to improve tissue- and/or cell-specific expression. For example, SV40 is a weaker promotor. As used herein, the term weak promotor indicates relatively little or infrequent RNA polymerase binding, resulting in lower transcription and low levels of the expressed protein. In combination with the selection marker, a weaker promotor enhances selection strength.

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In some embodiments, the expression vector and/or the second vector comprises at least one inverted terminal repeat (ITR) sequence. This mediates intermolecular recombination of genes.

20 In some embodiments, the expression vector and/or the second vector comprises a poly adenine region. A poly adenine (polyA) region encodes a poly adenine tail, a long chain of adenine nucleotides that increases the stability of mRNA.

In some embodiments, the expression vector and/or the second vector comprises a
25 transcriptional regulatory elements. Transcriptional regulatory elements are nucleotide sequences of a gene that are involved in regulation of genetic transcription. Advantageously, this may provide epigenetic control, for example preventing or limiting epigenetic inhibition during culturing of cells. An example of this is ubiquitous chromatin opening elements (UCOE).

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In some embodiments, the expression vector and/or the second vector may comprise a further selection marker, for example, puromycin or glutamine synthase or DHFR. In some embodiments, the selection marker is downstream of the promoter. This advantageously would be particularly appropriate for bispecific and other more complex molecules or

targets. In addition, these may be particularly advantages in systems with more than one vector.

5 In some embodiments, the expression vector and/or the second vector comprises inducible elements. Said inducible elements provide the advantage allowing cells to reach high density before turning on expression from gene constructs.

10 In some embodiments, the gene of interest and/or the exogenous nucleotide sequence encoding the at least an enzyme in the NAD⁺ synthesis pathway may be codon optimised. This is the process in which codons are changed from those which are rare in the host to those which are host-preferred. The codons in the gene of interest and/or the exogenous nucleotide sequence encoding the at least an enzyme in the NAD⁺ synthesis pathway may be selected to closely mimic the codon usage in the selected cell line, such that the frequency of codon usage in the gene is substantially the same as a gene which encodes the same or
15 similar protein in the cell line. This enhances heterologous expression levels.

20 In some embodiments, the cell line is a eukaryotic cell line. In some embodiments, the cell line is mammalian. This provides the advantage that the cell line provides the appropriate post-translational modifications to the expressed GOI.

In some embodiments, the cell line is plant, insect, yeast or prokaryotic.

25 In some embodiments, the cell line is selected from: Chinese hamster ovary (CHO) cells; HEK cells; and/or VERO cells. In some embodiments the CHO cells are CHO K1 cells, CHO DG44, CHO DXB11 or CHO-S cells. In some embodiments, the cell line are tumour cells; and/or CAR-T cells.

30 CHO cells exhibit a similar, inefficient metabolism as tumour cells, for example generating energy from glucose via anaerobic rather than aerobic pathways, utilising amino acids for energy and as biosynthetic skeletons to support rapid biosynthesis resulting in the generation of toxic metabolic biproducts. Therefore, metabolism in tumour cells would also reasonably be expected to be similar in CHO cells. CHO cells enjoy the benefit of being a good model for tumour cells, and use of the expression system in these cells is particularly advantageous.

In some embodiments, the cell line may be provided in suspension, adherent or in continuous culture perfusion mode. The cell line may be provided in the media formulation for example.

5 In some embodiments, the cell line is optimised to for the selection through feeding with an inhibitor, optionally a transferase inhibitor.

10 In some embodiments, the cell line is provided with an inhibitor, for example in the media formulation. The inhibitor is optionally an inhibitor as described herein. The inhibitor may inhibit the activity of the at least one enzyme in the NAD^+ synthesis pathway. The inhibitor may inhibit the activity of the endogenous and/or the exogenous at least one enzyme in the NAD^+ synthesis pathway. In some embodiments, the inhibitor inhibits the activity of endogenous or the exogenous at least one enzyme in the NAD^+ synthesis pathway more than exogenous or endogenous at least one enzyme in the NAD^+ synthesis pathway respectively. The activity may be inhibited as described herein.

15

In such embodiments, the cell line may comprise an endogenous nucleotide sequence which encodes at least one enzyme in the NAD^+ synthesis pathway. The inhibitor may inhibit the endogenous at least one enzyme in the NAD^+ synthesis pathway, reducing growth and/or survival of said cells. This means that cells comprising an exogenous at least one enzyme in the NAD^+ synthesis pathway express greater quantities of at least one enzyme in the NAD^+ synthesis pathway, restoring the pathway. Such cells also express the GOI and therefore the provision of an inhibitor selects for these cells.

20

The media formulation refers to the culture medium in which the cells are grown and survive.

25 In some embodiments, the media does not comprise Nicotinamide (NAM) and/or nicotinic acid.

In some embodiments, the media formulation does not comprise small molecular weight metabolites and/or amino acids that could serve as potential precursors of nicotinamide. This provides the advantage that the selection is more controlled. In some embodiments, the media formulation is substantially serum-free, protein-free and/or is tryptophan-free. In some embodiments, the media formulation is Ham's F12 + 8mM Glutamine culture medium. In some embodiments, the media formulation is supplemented with 10% (v/v) Foetal Bovine Serum. In some embodiments, the media is metabolite deficient. In some embodiments, the

30

medium is a commercially available protein free CHO medium such as CDCHO, CD FortiCHO or Excell.

5 In some embodiments, the media formulation comprises amino acids which support protein synthesis. For example, the media formation may comprise tryptophan.

10 The media formulation may be selected to be suitable for the growth and selection of the cell line. In some embodiments, the media formulation is optimised and feeds have been developed to further maximise selection and increase recombinant gene expression. In some embodiments, the media formulation comprises serum. This provides the advantages that viral vectors, such as lentiviral and AAV as described herein, operate with serum in the media.

15 The known industry standard CD-CHO medium contains 0.1-1.0 mg nicotinamide/mL. Other commercial media with publicly accessible compositions, for example DMEM, contain 1-4 mg nicotinamide/mL. The very earliest medium formulations developed by Ham in 1965 used nicotinamide and hence formulation of medium with nicotinamide became standard for all other generic media. Nicotinamide was probably preferred by Ham over nicotinic acid due to being supportive for cell growth or having greater stability in cell culture. The inventors have completed RNAseq experiments using several different CHO cell variants in the which show that transcript raw counts for NAMPT are ten times greater than those encoding NAPRT, further supporting this cellular advantage in the use of nicotinamide to support NAD synthesis.

25 In some embodiments, the media formulation is a basal medium. In such embodiments, the medium formulation further comprises a transferase inhibitor.

30 In some embodiments, wherein the media formulation further comprises an inhibitor as described herein. The inhibitor may be a transferase inhibitor as described herein.

In another aspect of the invention, a method for selecting cells comprising a gene of interest using the expression system, cell line, expression vector or media formulation is provided.

In another aspect of the invention, a method of culturing and/or expanding the cell line using the media formulation is provided. As discussed herein, expression of the at least one enzyme in the NAD⁺ synthesis pathway restores the ability of the cell to synthesise the key nutrient, allowing only those cells expressing the GOI to be
5 cultured and/or expanded.

In another aspect of the invention, a use of the expression system is provided, wherein cells that do not comprise at least one enzyme in the NAD⁺ synthesis pathway do not survive.

10 A skilled person will appreciate that all aspects of the invention, whether they relate to, for example, the composition, its use, or a method of treatment are equally applicable to all other aspects of the invention. In particular, aspects of the composition for example, may have been described in greater detail than in other aspects of the invention, for example, the use of the composition. However, the skilled person will appreciate where more detailed
15 information has been given for a particular aspect of the invention, this information is generally equally applicable to other aspects of the invention.

Examples

20 Materials:

CHO-K1 cell line was sourced from the European Collection of Authenticated Cell Cultures, ECACC (Cat No 85051005).

25 Cell culture medium, initial growth of cells in Ham's F12 + 8mM Glutamine (Sigma Merck) supplemented with 10% (v/v) Foetal Bovine Serum (New Zealand certified). Subsequent cell culture was performed in CD CHO medium (Gibco). A proprietary modified serum-free medium (devoid of nicotinamide, nicotinic acid and tryptophan) was synthesised.

30 Plasmids were synthesized by VectorBuilder. The first plasmid series was designed for EGFP expression, utilizing the mouse NAMPT gene (codon-optimized for CHO cells via SnapGene software) as a selection marker under the control of an SV40 promoter, with EGFP or IgG as the recombinant protein downstream of a cytomegalovirus (CMV) promoter. The second plasmid was designed for expressing the NAMPT protein, using the

same gene sequence, under the control of a CMV promoter, with puromycin as the selection marker downstream of an SV40 promoter.

Reagents (including target-designed guide RNAs) for CRISPR editing were purchased by
5 the University of Manchester Genome Editing Facility

Method summaries:

Development of a serum-free, suspension CHO-K1 master cell bank

10 Cells were adapted to suspension and serum-free growth by sequential step changes in the relative mixture of serum-containing (Hams F12) and serum-free (CD-CHO), supplemented with 0.1% (w/v) anti-clumping agent whilst growing the cells in shaking conditions. Over a period of 4 months cells went through step-down changes in the percentage of serum-containing medium until a serum-free cell line was harvested. At this stage a master cell
15 bank was deposited.

Transfection procedures

For each transfection reaction approximately 4×10^7 cells were harvested via centrifugation at 100g for 5 minutes and washed with cold PBS. The cells were re-centrifuged and pellets
20 were resuspended in 950 μ L PBS. Three electroporation cuvettes were prepared, two containing 20 μ g plasmid, and one with sterile water as a control. A 950 μ L cell-PBS suspension was added to each cuvette and the mixtures were incubated on ice for 5 minutes. Transfection was conducted by electroporation, using Bio-Rad Gene Pulser Xcell system. Two consecutive pulses (300 V, 950 μ F, and 20 ms) were applied to each DNA/cell mixture.
25 Cells were then placed on ice for 5 minutes and transferred to CD CHO medium, in the absence or presence of added nicotinic acid (50nM). For transient transfection studies were performed over a period of 7 days. For stable transfections, sub-culture was performed every 3-4 days, diluting cell numbers and replacing with the relevant CD-CHO medium type.

Analytical procedures

A variety of standard and in-house methods were applied to assessment of cell culture.

Cell density and viability, metabolite profiles (enzymatic, GC-MS, LC-MS), karyotyping, flow cytometry, RNA isolation, characterisation and assessments (PCR, qRT-PCR), protein isolation and assessment (western blotting, ELISA)

5 Results:

The applicant has shown that NAMPT CRISPR gene knockout cells do not grow without nicotinic acid. Fig. 4 and 5 show that the transferase inhibitor of NAMPT (FK866) is lethal to CHO-K1 cells. The fatal effect of NAMPT transferase inhibitor was overcome by the
10 presence of nicotinic acid. The basal medium contains nicotinamide. This illustrates the central importance of NAMPT (and NAD⁺) to cell health.

Fig. 6 and 7 show that genetic knock-out of NAMPT in the cell line generates cells that cannot survive in the absence of nicotinic acid. The CRISPR NAMPT knockout is lethal to
15 cells. This is prevented with nicotinic acid.

Two different knock-out gRNA sequences used in Fig. 6 and 7. In Fig 6, CRISPR sg1-3 cell line was maintained in CD CHO medium, supplemented with nicotinic acid (50 µM). At time zero (t=0), the medium was switched to that with or without nicotinic acid. The control
20 cells were maintained in basal medium (containing nicotinamide) throughout. Where added, the FK866 concentration was 50 nM.

In Fig. 7, CRISPR sg2 cell line was maintained in CD CHO medium, supplemented with nicotinic acid (50 µM). At time zero (t=0), the medium was switched to that with or without
25 nicotinic acid. The control cells were maintained in basal medium (containing nicotinamide) throughout. Where added, the FK866 concentration was 50 nM.

Figure 8 shows a proprietary expression vector was generated as an example using GFP expression and herceptin biosimilar molecule.

30

Figure 9 shows IgG expression from NAMPT vector in transient and stable transfection. This stable and transient expression of the supports strong expression of a Herceptin biosimilar in the host CHO-K1 cell line and in both genetic knock-out cell lines. The * symbol denotes that medium samples were collected on day 7 of batch growth for transient

transfection. The ** symbol denotes that cells were in T75 flasks for 5 days in 10 mL media and then supplemented with an additional 10 mL fresh medium, for stable transfection. Medium samples for able transfections were obtained 2 days after the medium supplementation. Each well was loaded with 30 μ L of undiluted medium samples.

5

Fig. 10 shows a diagram of the vector transfected into the cells in Fig. 9.

Figure 11 shows an exemplary embodiment of the mechanism of the invention. In this embodiment, the vector from Figure 10 was transfected into cells to produce a recombinant
10 NAMPT KO cell line.

Figure 12 shows protein expression levels from a knockout transfected with a gene of interest under NAMPT selection, using the mechanism shown in Figure 11. In this embodiment, batch cultures were grown in CDCHO media. Fed-batch cultures had a commercial feed
15 added during culture to augment nutrients consumed by the cells.

Figure 13 shows another exemplary embodiment of the mechanism of the invention. In this embodiment, the vector from Figure 10 was transfected into cells to produce a recombinant NAMPT KO cell line. The inhibitor FK866 was added to apply selection pressure to the
20 cells.

Figure 14A to D show that the addition of the inhibitor FK866 applies selection pressure to result in amplification of protein expression. In these experiments, a comparison was carried out between non treated cells and cells that were treated with 5nM FK866 inhibitor. From
25 the results of the experiments, it can be seen that the amount of recombinant protein produced following inhibition with FK866 was greater than when the cells that were untreated. For example, the volumetric titer that was obtained following treatment of cells with 5mM FK866 was up to 4000 mg/L compared with up to 2000 mg/L for untreated cells. The use of an inhibitor to amplify selection and hence expression highlights the power of the
30 expression system. Advantageously, enzyme inhibition results in increased gene expression, showing the obligate nature of the enzyme.

Thus, the applicant has shown that NAMPT is a selection marker that drives high protein expression with reduced screening effort. The volumetric titres that can be obtained using this marker can be up to 4 g/L and the productivity can be up to 50 pcd.

The applicant has also shown that NAMPT is a more powerful selective marker when compared to other previous markers such as Glutamine Synthase (GS) which can be bypassed by other amino acids. The functions of glutamine for cells such as for energy generation and biosynthesis can be alleviated by other amino acids. Aspartic and asparagine can also be used to replace energy generation bypassing glutamine. Cells grown in Glutamine free medium under the GS expression system significantly increase the metabolism of a panel of amino acids highlighting that cells bypass GS. In contrast in the absence of NAMPT/ nicotinic acid and / or nicotinamide there is no alternative pathway in CHO so cell death is immediate.

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Sequences

SEQ ID 1: NAMPT

>sp|P43490|NAMPT_HUMAN Nicotinamide phosphoribosyltransferase OS=Homo sapiens OX=9606 GN=NAMPT PE=1 SV=1

MNPAAEAEFNILLATDSYKVTHYKQYPPNTSKVYSYFECREKKTENSKLRKVKEY
ETV FY
GLQYILNKYLKGKVVTKEKIQEAKDVYKEHFQDDVFNEKGWNYILEKYDGHLP
IE
IKAVP
5 EGFVIPRGNVLTVENTDPECYWLTNWIETILVQSWYPITVATNSREQKKILAKYL
LETS
GNLDGLEKYLHDFGYRGVSSQETAGIGASAHLVNFKGTDTVAGLALIKKYYGTK
DPVPGY
SVPAAEHSTITAWGKDHEKDAFEHIVTQFSSVPVSVVSDSYDIYNACEKIWGEDLR
10 HLIV
SRSTQAPLIIRPDSGNPLDTVLKVLEILGKKFPVTENSKGYKLLPPYLRVIQGDGVDI
NT
LQEIVEGMKQKMWSIENIAFGSGGGLLQKLTRDLLNCSFKCSYVVTNGLGINVFK
DPVAD
15 PNKRSKKGRLSLHRTPAGNFVTLEEGKGDLEEYQDLLHTVFKNGKVTKSYSFDE
IRKNA
QLNIELEAAHH

SEQ ID 2: NAPRT

20 >sp|Q6XQN6|PNCB_HUMAN Nicotinate phosphoribosyltransferase OS=Homo sapiens
OX=9606 GN=NAPRT PE=1 SV=2
MAAEQDPEARAAARPLLTDLYQATMALGYWRAGRARDAAEFELFFRRCPPFGGAF
ALAAGL
RDCVRFLRAFRLRDADVQFLASVLPPDTPAFFEHLRALDCSEVTVRALPEGSLAF
25 PGVP
LLQVSGPLLVVQLLETPLLCLVSYASLVATNAARLRRIAGPEKRLLEMGLRRAQGP
DGGL
TASTYSYLGGFDDSSSNVLAGQLRGVPVAGTLAHSFVTSFSGSEVPPDPMLAPAAGE
GPGV
30 DLAACAQVWLEQVCAHLGLGVQEPHPGERAAAFVAYALAFPRAFQGLLDTYSVW
RSGLPNF
LAVALALGELGYRAVGVRLD SGDLLQQAQEIRKVFRAAAAQFQVPWLESVLIVVS
NNIDE

EALARLAQEGSEVNVIGIGTSVVTCPQQPSLGGVYKLVAVGGQPRMKLTEDPEKQ
TLPGS

KAAFRLGSDGSPLMDMLQLAEEPVPQAGQELRVWPPGAQEPCTVRPAQVEPLL
RLCLQQ

5 GQLCEPLPSLAESRALAQLSLSRLSPEHRRLRSPAQYQVVLSERLQALVNSLCAGQ
SP

SEQ ID 3: QPRT

>sp|Q15274|NADC_HUMAN Nicotinate-nucleotide pyrophosphorylase [carboxylating]

10 OS=Homo sapiens OX=9606 GN=QPRT PE=1 SV=3

MDAEG LALLP PVT L AAL VDSWL REDCPGLNYAALVSGAGPSQAALWAKSPGVL
AGQPFF

DAIFTQLNCQVSWFLPEGSKLVPVARVAEVRGPAHCLLLGERVALNTLARCSGIAS
AAAA

15 AVEAARGAGWTGHVAGTRKTTPGFRLVEKYGLLVGGAASHRYDLGGLVMVKD
NHVVAAGG

VEKAVRAARQAADF TLKVEVECSSLQEAVQAAEAGADLVLLDNFKPEELHPTAT
VLKAQF

PSVAVEASGGITLDNLPQFCGPHIDVISMGMLTQAAPALDFSLKLFKEVAPVPKI

20 H

SEQ ID 4: IDO

>sp|Q6ZQW0|I23O2_HUMAN Indoleamine 2,3-dioxygenase 2 OS=Homo sapiens
OX=9606 GN=IDO2 PE=1 SV=5

25 MEPHRPNVKTAVPLSLESYHISEEYGFLLPDSLKELPDHYRPWMEIANKLPQLIDA
HQLQ

AHVDKMPLLSCQFLKGHREQRLAHLVLSFLTMGYVWQEGEAQPAEVLPRNLALP
FVEVSR

NLGLPPILVHSDLVLTNWTKKDPDGFLEIGNLETIISFPGGESLHGFILVTALVEKEA

30 VP

GIKALVQATNAILQPNQEALLQALQRLRLSIQDITKTLGQMHDYVDPDIFYAGIRIF
LSG

WKDNPAMPAGLMYEGVSQEPLKYSGGSAAQSTVLHAFDEFLGIRHSKESGDFLY
RMRDYM

PPSHKAFIEDIHSAPSLRDYILSSGQDHLLTAYNQCVQALAE LRSYHITMVT KYLIT
AAA
KAKH GKPNHLP GPPQAL KDRGTGGTAVMSFLKSVRDKTLESILHPRG

5 SEQ ID 5: TDO

>sp|P48775|T23O_HUMAN Tryptophan 2,3-dioxygenase OS=Homo sapiens OX=9606
GN=TDO2 PE=1 SV=1

MSGCPFLGNNFGYTFFKKLPVEGSEEDKSQTGVNRASKGGLIYGNYLHLEKVLNAQ
ELQSE

10 TKGNKIHD EHLFIITHQAYELWFKQILWELDSVREIFQNGHVRDERNMLKVVS RM
HRVSV
ILKLLVQQFSILETMTALDFNDFREYLS PASGFQSLQFRLL ENKIGVLQNM RVPYN
RRHY

RDNFKGEENELLLKSEQEKTLELVEAWLERTPGLEPHGFNFWGKLEKNITRGLEE

15 EFIR

IQAKEESEEEKEEQVAEFQKQKEVLLSLFDEKRHEHLLSKGERRLSYRALQGALMIY
FYRE

EPRFQVPFQLLTSLMDIDSLMTKWRYNHVCMVHRMLGSKAGTGGSSGYHYLRST
VSDRYK

20 VFVDL FNLSTYLIPRHWIPKMNP TIHKFLYTA EYCDSSYFSSDESD

SEQ ID 6: NMNA1

>sp|Q9HAN9|NMNA1_HUMAN Nicotinamide/nicotinic acid mononucleotide
adenylyltransferase 1 OS=Homo sapiens OX=9606 GN=NMNAT1 PE=1 SV=1

25 MENSEKTEVVLLACGSFNPITNMHLRLFELAKDYMN GTGRYTVVKGIISP VGDAY
KKKGL

IPAYHRVIMAE LATKNSKWVEVDTWESLQKEWKETLKVLRHHQEKLEASDCDHQ
QNSPTL

ERPGRKRKWTETQDSSQKKSLEPKTKAVPKVKLLCGADLLESFAVPNLWKS EDIT

30 QIVAN

YGLICVTRAGNDAQKFIYESDVLWKHRSNIHVVNEWIANDISSTKIRRALRRGQSI
RYLV

PDLVQEYIEKHNL YSSESEDRNAGVILAPLQRNTAEAKT

SEQ ID 7: NMNA2

>sp|Q9BZQ4|NMNA2_HUMAN Nicotinamide/nicotinic acid mononucleotide
adenylyltransferase 2 OS=Homo sapiens OX=9606 GN=NMNAT2 PE=1 SV=1

MTETTKTHVILLACGSFNPITKGHIQMFERARDYLHKTGRFIVIGGIVSPVHDSY GK

5 QGL

VSSRHLIMCQLAVQNSDWIRVDPWECYQDTWQTTCVLEHHRDLMKRVTGCIL
SNVNTPSMTPVIGQPQNETPQPIYQNSNVATKPTAAKILGKVGESLSRICCVRPPVERFTFVD
ENA10 NLGTVMRYEEIELRILLCGSDLLESFCIPGLWNEADMEVIVGDFGIVVPRDAAD
TDRIMNHSSILRKYKNNIMVVKDDINHPMSVVSSTKSRLALQHGDGHVVDYLSQPVIDY
ILKSQ

LYINASG

15

SEQ ID 8: NMNA3

>sp|Q96T66|NMNA3_HUMAN Nicotinamide/nicotinic acid mononucleotide
adenylyltransferase 3 OS=Homo sapiens OX=9606 GN=NMNAT3 PE=1 SV=2

MKSRIPVVLLACGSFNPITNMHLRMFEVARDHLHQTGMVYQVIQGIISPVNDTYGK

20 KDLAA

SHHRVAMARLALQTSWIRVDPWESEQAQWMETVKVLRHHHSKLLRSPQMEG
PDHGKALFSTPAAVPELKLLCGADV LKTFQTPNLWKDAHIQEIVEKFGLVCVGRVGHDPKGY
IAESP25 ILRMHQHNIHLAKEPVQNEISATYIRRALGQGQSVKYLPDAVITYIKDHGLYTKGS
TWK

GKSTQSTEGKTS

Claims

1. An expression system for selecting the presence of a gene of interest, the expression
5 system comprising:
- (i) a cell line wherein the activity of at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited,
the cell line optionally further comprising the gene of interest, and the cell line optionally lacking an endogenous nucleotide sequence which encodes the at least one
10 enzyme in the NAD⁺ synthesis pathway;
 - (ii) an expression vector comprising an exogenous nucleotide sequence which encodes the at least one enzyme in the NAD⁺ synthesis pathway; and
 - (iii) a media formulation which does not comprise nicotinamide (NAM) and/or nicotinic acid.
- 15
2. A cell line wherein the activity of at least one enzyme in the NAD⁺ synthesis pathway is at least partially inhibited, optionally further comprising a gene of interest, and optionally lacking an endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway.
- 20
3. An expression vector comprising an exogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway and optionally a gene of interest.
4. A media formulation which does not comprise nicotinamide (NAM) and /or nicotinic
25 acid.
5. The expression system or cell line or expression vector according to any of claims 1 to 3, wherein the gene of interest is encoded by the expression vector and/or by a second vector.
- 30
6. The expression system or cell line according to any one of claims 1 to 3 or 5, wherein the cell line comprises the expression vector.

7. The expression system, cell line or expression vector according to any one of claims 1 to 3 or 5 to 6, wherein the at least one enzyme in the NAD⁺ synthesis pathway is selected from nicotinamide phosphoribosyltransferase (NAMPT), nicotinic acid phosphoribosyltransferase (NPRT) and/or quinolinic acid phosphoryltransferase (QPRT).
- 5
8. The expression system, cell line, or expression vector according to claim 7, wherein the at least one enzyme in the NAD⁺ synthesis pathway is a heterologous mammalian NAMPT.
- 10
9. The expression system, cell line, or expression vector according to claim 8, wherein the at least one enzyme in the NAD⁺ synthesis pathway has at least 70% sequence identity to SEQ ID 1 to 8.
10. The expression system, cell line, or expression vector according to any one of claims 1 to 3 or 5 to 9, wherein the gene of interest encodes a recombinant protein, viral vector, component of a viral vector, viral vaccine, intracellular protein and/or receptor.
- 15
11. The expression system, cell line, or expression vector according to any one of claims 1 to 3 or 5 to 10, wherein the exogenous nucleotide sequence encoding the at least an enzyme in the NAD⁺ synthesis pathway is under the control of a promoter, optionally wherein the promoter is CMV, SV40 and/or mPGK.
- 20
12. The expression system, cell line, or expression vector according to any one of claims 1 to 3 or 5 to 11, wherein the gene of interest is under the control of a promoter, optionally wherein the promoter is CMV, SV40 and/or mPGK.
- 25
13. The expression system or cell line according to any one of claims 1 to 2 or 5 to 12, wherein the cell line is a eukaryotic cell line, optionally, selected from: Chinese hamster ovary K1 cells; HEK cells; and/or VERO cells.
- 30
14. The expression system or cell line according to any one of claims 1 to 2 or 5 to 13, wherein the cell line is genetically engineered to lack the endogenous nucleotide sequence which encodes at least one enzyme in the NAD⁺ synthesis pathway.

15. The expression system or media formulation according to any one of claims 1 or 4 to 14, wherein the media formulation further comprises an inhibitor, optionally wherein the inhibitor is a transferase inhibitor.

5 16. The expression system or media formulation according to claim 15, wherein the transferase inhibitor is a Nicotinamide Phosphoribosyl Transferase (NAMPT) Inhibitor.

17. The expression system or media formulation according to any one of claims 1 or 4 to 16, wherein the media formulation is a substantially serum-free and/or protein-free medium.

10

18. A method for selecting cells comprising a gene of interest using the expression system, cell line, expression vector or media formulation according to any one of claims 1 to 17.

15 19. A method of culturing and/or expanding the cell line according to any one of claims 1, 2, 5 to 14 using the media formulation according to any one of claims 4 and 15 to 17.

20. Use of the expression system according to any one of claims 1 to 17, wherein cells that do not comprise at least one enzyme in the NAD⁺ synthesis pathway do not survive.

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