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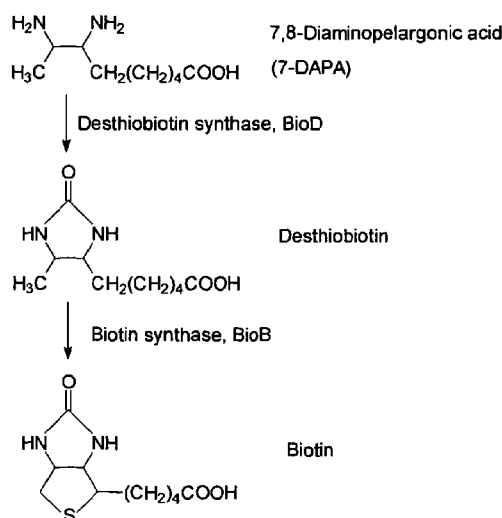
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(54) Title: CELLS FOR THE PRODUCTION OF BIOTIN - TAGGED VIRAL VECTORS OR PROTEINS



(57) Abstract: The present invention provides a cell for the production of a viral vector or protein of interest (POI), which cell is capable of converting 7,8-Diaminopelargonic acid (7-DAPA) or 7-keto-8-aminopelargonic acid (7-KAPA) into biotin or desthiobiotin, thus producing a biotin/desthiobiotin-tagged viral vector/POI. The present invention also provides methods/plasmids for the production of such cells, the use of such cells to produce biotin/desthiobiotin-tagged viral vectors/POIs and methods for their purification.

CELLS FOR THE PRODUCTION OF BIOTIN-TAGGED VIRAL VECTORS OR PROTEINS

FIELD OF THE INVENTION

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The present invention relates to cells and methods for the production of tagged viral vectors and proteins. In particular it relates to methods for the production of biotin- or desthiobiotin-tagged viral vectors, proteins and cells.

10 BACKGROUND TO THE INVENTION

Viral vector production

15 Lentiviral vectors are attractive for gene therapy applications, but the technology is associated with some problems. For example, the progress in lentiviral gene therapy has been hampered by the requirement for production of purified lentiviral vectors with high titre. Viral vectors are often purified using conventional multi-step downstream processing methods including ultracentrifugation, precipitation,
20 filtration and ion-exchange chromatography. These low specificity processes are time-consuming and some are scale limited and generally result in low recoveries of infectious viral particles. Antibody and heparin affinity chromatography have enabled a specific separation of adeno-associated viruses and herpes simplex viruses from impurities generated by host cells. However, both of these techniques
25 have potential constraints in manufacturing. Antibody production, purification and immobilisation are costly at large scale and for each virus type a different antibody would be required. In addition, many cellular proteins are known to associate with heparin, hence the use of a heparin column requires the incorporation of a specific step to remove contaminating heparin-binding proteins.

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Affinity chromatography may be used as a single-step capture method for the generic recovery of viral vectors by exploiting streptavidin and biotin interactions. Nesbeth *et al.* (Molecular Therapy 2006, 13, 814-822) engineered a novel human 293T based packaging cell line BL15, which metabolically produces spontaneously

biotin-tagged lentiviral vectors requiring only biotin in the culture medium. This metabolic biotinylation technology facilitates highly efficient affinity-mediated paramagnetic-particle and chromatographic capture of viral particles.

- 5 A similar system has been described for adenovirus (Parrott *et al* (2003) Mol. Ther. 8:688-700), in which the fiber capsid protein is genetically fused to a biotin acceptor peptide, which is metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells.
- 10 However, the value of such biotinylation systems for purification of viral vectors in manufacturing is limited because the crude supernatants containing biotinylated viruses are contaminated with competing free-biotin that reduce the efficiency of viral capture, meaning that a medium change prior to viral harvest is obligatory (Nesbeth *et al.* 2006 (as above); Williams *et al* (2005) Biotechnology and
- 15 Bioengineering 89: 783-787; and Williams *et al* (2005) Journal of Chromatography B 820: 111-119).;

There is thus a need for improved packaging cell lines which address the above-mentioned problem.

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Recombinant protein production

- The concept of using biotin-tagging has also been described for the recovery of recombinant proteins. For example, US 2004/0033603 relates to a eukaryotic
- 25 expression system for the *in vivo* biotinylation of a protein. Biotin-tagged recombinant proteins have also been produced in mammalian cells using a biotin acceptor tag and the endogenous biotinylation system of the cell (Parrott and Barry (2000) Mol. Ther. 1:96-104).

- 30 However, such technology suffers from a similar drawback to the production of biotin-tagged viral vectors in that the crude supernatants containing biotinylated

protein are contaminated with competing free-biotin that reduce the efficiency of protein purification.

There is thus a need for improved methods for the production and purification of recombinant proteins which address the above-mentioned problem.

Viral clearance

Recombinant monoclonal antibodies (Mabs) continue to increase in importance as therapeutics in the treatment of cancer and autoimmune diseases. However, large-scale production of Mabs still remains a challenge. The commercial manufacturing procedure must delivery a reliably high yield with downstream high product throughput operations to produce a pure product.

Various mammalian cell lines have been described as efficient expression systems for the production of Mabs. However, mammalian cells systems are susceptible to contamination with adventitious viruses that may be introduced through raw materials or failures in process controls. It is thus necessary to include a "viral clearance" procedure, in order to remove such contaminants from the product preparation.

The Mab large-scale purification process is usually built around the use of immobilized Protein A as the primary antibody capture step, followed by three or four further purification units and two or three "polishing" purification units to remove product-related impurities such a cell lysis components. A degree of viral clearance is obtained by both the capture and polishing steps.

Methods directed specifically at viral clearance divide into those based on inactivation, such as using low pH or ultraviolet irradiation; and those based on removal, such as Q membrane chromatography, nanometer filtration and depth filtration.

Inactivation-based methods have drawbacks in that they are not 100% effective, opening up the possibility of active virus surviving into the product preparation. The techniques used can also have an adverse effect on the product itself. For example, the low pH may alter the biological activity of the Mab therapeutic or
5 cause protein aggregation of low pH-sensitive Mabs. UV irradiation can also cause partial denaturation of some Mabs. Finally, even if the contaminating virus is successfully inactivated by the procedure, the inactivated virus may cause unwanted effects (complexing of product, induction of immune responses) if left in the product preparation, or require a further purification step if the inactivated virus
10 is to be removed.

Removal-based mechanisms may not be effective against all viruses, as viruses differ considerably in their size, structure and binding activities.

15 There is thus a need for improved methods for viral clearance, for example during the large-scale production and purification of therapeutic antibodies.

SUMMARY OF ASPECTS OF THE INVENTION

20 The present inventors have now developed new packaging cell lines, which can be used to produce biotin-tagged or desthiobiotin-tagged viral vectors in the presence of 7,8-diaminopelargonic acid (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA). These systems have a significant advantage over the known biotin-tagging packaging cells which metabolically label vectors in the presence of free biotin.
25 The biotin precursor molecules, 7-DAPA and 7-KAPA do not bind avidin/streptavidin, so when a tagged viral vector is produced using the cells of the present invention, there is no free biotin in the culture medium. This means that the tagged vector can be captured directly from crude supernatants without the need for pre-treatment to remove free avidin-binding impurities.

30 The same advantage may also be realised by using similar technology for purification of expressed proteins. In other words, biotin- or desthiobiotin-tagged

proteins may be captured directly after synthesis without the need for removal of avidin-binding contaminants if they are produced by a cell line capable of using a biotin precursor molecule such as 7-DAPA or 7-KAPA.

5 Moreover, similar technology offers an alternative to and a significant advantage over existing viral clearance techniques. For example, in the case of a hybridoma cell line for antibody production, if a cell line is used which is capable of labelling viruses with biotin/desthiobiotin in the presence of 7-DAPA or 7-KAPA, then any adventitious enveloped virus produced by the cells will be tagged, but the antibody
10 will not. The virus may therefore be removed using streptavidin/avidin, such as by passage through an avidin/streptavidin functionalised filter. The absence of biotin in the culture medium avoids competition by free biotin, adding to the efficiency of the procedure. Another advantage of this technique is that, as all adventitious agents that acquire the biotin/desthiobiotin presenting membrane proteins as they
15 bud from the cell will be tagged, they will all be removed by the filtration process regardless of their size or identity. It is thus unnecessary to identify the type(s) of contaminating enveloped virus prior to viral clearance.

Thus in a first aspect, the present invention provides a producer cell for the
20 production of a viral vector, which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, thus producing a biotin- or desthiobiotin-tagged viral vector.

The producer cell may produce a desthiobiotin-tagged viral vector. The high-
25 affinity binding of biotin to streptavidin (dissociation constant $K_d \sim 10^{-15}$ M) makes it unsuitable for processes requiring efficient elution of viruses from affinity supports under physiological conditions (Williams *et al.*, 2005, as above). Since desthiobiotin has a lower affinity for avidin/streptavidin ($K_d \sim 10^{-13}$ M) than biotin, desthiobiotin-labelled viral vectors are amenable to gentle elution strategies.

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The viral vector may be a retroviral vector, such as a lentiviral vector.

The producer cell according may produce membrane-bound biotin/desthiobiotin.

The producer cell may express a biotin/desthiobiotin acceptor sequence on its surface, for example as part of an endogenous or exogenous cell-membrane protein.

5

The cell may be stably transfected with the desthiobiotin/biotin-producing enzyme(s) so that it is only necessary to introduce the vector-encoding sequences to produce the viral vector.

10 Alternatively the cell may be stably transfected with the desthiobiotin/biotin-producing enzyme(s) and vector encoding sequences, such that the cell lines constitutively produce the particular vector.

In a second embodiment of this first aspect of the invention, there is provided a cell
15 for the production of a protein of interest (POI), which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin/desthiobiotin, thus producing a biotin- or desthiobiotin-tagged POI.

The cell may express a fusion protein containing the POI and a biotin/desthiobiotin
20 acceptor peptide.

In a third embodiment of this first aspect of the invention, there is provided a cell for the production of an untagged entity of interest (EOI), which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-
25 KAPA) into biotin or desthiobiotin, such that an adventitious agents produced by the cell will be biotin/desthiobiotin-tagged and may therefore be distinguished from the EOI.

For example, the cell of the third embodiment may be a hybridoma cell line for the
30 production of an antibody. Since the antibody produced by the cell are not tagged, but adventitious agents (such as viruses) produced by the cell are tagged with biotin/desthiobiotin, this facilitates viral clearance of the antibody preparation.

In this embodiment the cell may be capable of biotin-tagging an adventitious agent. As mentioned above, biotin has a higher affinity for avidin/streptavidin than desthiobiotin. Since it is not necessary or desirable to elute the adventitious agent (e.g. virus) once cleared, it may be preferable to use biotin for increased efficiency of capture and removal from the preparation comprising the EOI. Again, the absence of biotin in the culture media avoids contamination by free biotin, adding to the efficiency of the procedure.

In a fourth embodiment of this first aspect of the invention, there is provided a cell which is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, such that in the presence of 7-DAPA or 7-KAPA, the cell produces biotin/desthiobiotin-tagged cell surface proteins.

The cell of the fourth embodiment of the invention may be coated with desthiobiotin or biotin making it useful for a variety of techniques, such as magnetic labelling with paramagnetic/streptavidin/avidin beads, for use in cell-patterning, tissue culturing, cell-screening and in vivo cell-tracking; or labelling with or attachment to other moieties such as fluorescent labels and solid supports.

The choice of desthiobiotin/biotin modification will depend on the intended use of the cell and whether avidin/streptavidin mediated manipulation is desired in a reversible manner (in which case desthiobiotinylation may be used) or virtually irreversible manner (in which case biotinylation may be used).

For all four embodiments of this first aspect of the invention, the cell may express an enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin, such as the E. Coli BioD protein or a homolog thereof.

The cell may express two enzymes:

a first enzyme capable of catalysing the conversion of 7-keto-8-aminopelargonate (7-KAPA) into 7,8-Diaminoperlargonate (7-DAPA); and

a second enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin

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For example, the first enzyme may be BIO3 or a homolog thereof and the second enzyme may be BIO1 or a homolog thereof, so the cell may express a BIO3-BIO1 fusion protein. BIO3 and BIO1 are derivable from *Arabidopsis Thaliana*.

10 The cell may express three enzymes:

a first enzyme capable of catalysing the conversion of 7-keto-8-aminopelargonate (7-KAPA) into 7,8-Diaminoperlargonate (7-DAPA);

a second enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin; and

15 a third enzyme capable of catalysing the conversion of desthiobiotin into biotin.

For example, the first enzyme may be BioA or a homolog thereof and the second enzyme may be BioD or a homolog thereof, the third enzyme may be BioB or a
20 homolog thereof. The cell may, for example, express a BioA-BioD-BioB fusion protein.

In connection with the first, third and fourth embodiments of the first aspect of the invention, the cell may comprise a biotin/desthiobiotin acceptor sequence as part of
25 or fused to cell membrane protein (for example an exogenous cell membrane protein). This gives a surface protein marker with the capacity to be biotinylated/desthiobiotinylated, to produce biotin/desthiobiotin-tagged virus as it buds from the cell and/or biotin/desthiobiotin-labelling the cell itself.

30 The cell may additionally express a polyhistidine tag, resulting in the production of a polyhistidine/desthiobiotin dual tagged viral vector/POI. This may be achieved

by a fusion protein comprising the desthiobiotin acceptor sequence and the histidine tag, or they may be expressed on separate proteins.

The cell of the first aspect of the invention may express a ligase enzyme which couples biotin/desthiobiotin to a biotin/desthiobiotin acceptor peptide, such as BirA or a homolog thereof.

In a second aspect, the present invention provides a method for producing a cell according to the first aspect of the invention, which comprises the step of introducing a nucleotide sequence which encodes an enzyme which catalyses the conversion of 7,8-Diaminoperlargonate (7-DAPA) or (7-KAPA) into desthiobiotin into the cell.

In a third aspect, there is provided a method for producing a desthiobiotin-tagged viral vector which comprises the step of production of the viral vector using a producer cell according to the first aspect of the invention in the presence of 7-DAPA or 7-KAPA.

In a second embodiment of this aspect, there is provided a method for producing a desthiobiotin/biotin-tagged protein of interest (POI) which comprises the step of expression of the POI from a cell according to the second embodiment of the first aspect of the invention in the presence of 7-DAPA or 7-KAPA.

In a fourth aspect, there is provided a method for the purification of a viral vector/protein of interest (POI), which comprises the following steps:

(i) producing a viral vector / POI by a method according to the third aspect of the invention;

(ii) avidin-based capture of the vector/POI; and

(iii) elution of the captured vector/POI.

The vector/POI may be purified directly following production, so there is no washing step between steps (i) and (ii).

The 7-DAPA/7-KAPA concentration and/or incubation time may be selected in order to optimise the number of desthiobiotin molecules on the surface of viral particles for subsequent recovery.

5

In a fifth aspect, the present invention provides a kit for the production of a cell according to the first aspect of the invention, which comprises:

(i) a plasmid comprising a nucleotide sequence encoding an enzyme capable of synthesising desthiobiotin from 7-DAPA, such as bioD, BIO3 or a homolog or
10 ortholog thereof;

and any one or more of the following:

(ii) a plasmid comprising a nucleotide sequence encoding an enzyme capable of converting 7-KAPA to 7-DAPA, such as bioA, BIO1 or a homolog or ortholog thereof;

15 (iii) a plasmid comprising a nucleotide sequence encoding an enzyme capable of converting desthiobiotin to biotin, such as bioB, BIO2 or a homolog or ortholog thereof; and

(iv) a plasmid comprising a nucleotide sequence encoding a ligase enzyme capable of covalently coupling desthiobiotin to an acceptor site, such as BirA or a
20 homolog or ortholog thereof.

In connection with the first embodiment of the first aspect of the invention there is provided a kit for the production of a producer cell which also comprises any one or more of the following:

25 (v) a plasmid comprising a viral genome;

(vi) one or more plasmids encoding proteins required by the producer cell in order to produce a viral particle, such as Gag, Pol and/or Env;

(vii) a plasmid encoding a desthiobiotin acceptor sequence, optionally as part of or fused to an exogenous cell membrane protein; and

30 (viii) a plasmid encoding a His₆ sequence, optionally as part of or fused to an exogenous cell membrane protein.

In connection with the second embodiment of the first aspect of the invention there is provided a kit which also comprises:

(v) a plasmid comprising a nucleotide sequence encoding the POI, optionally joined to a nucleotide sequence encoding a desthiobiotin acceptor and/or
5 a nucleotide sequence encoding a His₆ sequence.

In a sixth aspect, the present invention provides a producer cell for producing a viral vector which comprises a desthiobiotin-tagged cell surface protein, which co-packages with the viral vector causing the viral vector produced by the cell to be
10 desthiobiotin-tagged.

In a seventh aspect, the present invention provides a method for purification of an entity of interest (EOI) which comprises the following steps:

(i) production of the EOI using a cell according to the third embodiment of the first aspect of the invention, in the presence of 7-DAPA or 7-
15 KAPA; and

(ii) avidin-based removal of the tagged adventitious agent.

The EOI may be an antibody, the cell may be a hybridoma cell line and the adventitious agent may be an enveloped virus.
20

In an eighth aspect, the present invention provides a method for producing a biotin- or desthiobiotin-coated cell, which comprises the step of culturing a cell according to claim 4 in the presence of 7-DAPA or 7-KAPA.

25 In a ninth aspect, the present invention provides biotin- or desthiobiotin-coated cell according to the fourth embodiment of the first aspect of the invention. The cell may be made by a method according to the eighth aspect of the invention.

The cell may be labelled with paramagnetic avidin/streptavidin beads or a
30 fluorescent label or attached to a solid support.

In a tenth aspect there is provided use of a cell according to the ninth aspect of the invention in cell patterning, tissue culturing, cell screening or cell tracking.

DESCRIPTION OF THE FIGURES

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Fig. 1. The biosynthetic pathway of biotin.

Fig. 2. Synthetic scheme of 7,8-diaminopelargonic acid (7-DAPA).

Fig. 3. ^{13}C -NMR spectra of (A) desthiobiotin in d_6 -DMSO and (B) 7-DAPA in D_2O .

10 **Fig. 4.** Laser scanning confocal microscopy images of DBL cells and the control BL15 cells incubated with 2 mM 7-DAPA for 96 h. (A) No detectable fluorescence on BL15 cells after avidin-FITC staining. (B) Desthiobiotin detected on the surface of DBL cells after avidin-FITC staining. (C) DBL cell membranes visualised by CellMask[™] staining. (D) Overlay of B and C.

15 **Fig. 5.** Characterisation of the extent of desthiobiotinylation on the surface of DBL cells by using the HABA-avidin assay. (A) 7-DAPA concentration-dependent desthiobiotinylation after 96 h. (B) Incubation duration-dependent desthiobiotinylation of DBL cells contacted with 1 mM DAPA. The error bars represent standard deviations of triplicate samples

20 **Fig. 6.** (A) The influence of the concentration of 7-DAPA contacted with DBL cells for 96 h upon the capture of desthiobiotinylated VSV-G pseudotyped lentiviral vectors on Streptavidin MagneSpheres[®]. (B) Elution efficiencies of the lentiviral vectors produced by DBL cells incubated with 7-DAPA at 0.5 (blank columns) and 2 mM (grey columns) by using different concentrations of biotin. 7-DAPA-containing lentiviral supernatant (3 mL) from cultures of DBL cells was contacted with 100 μL of Streptavidin MagneSpheres[®] for 2 h at room temperature using a rotary mixer. Samples (100 μL) were taken from the viral supernatants before and after the contact period and assayed to infect TE671 cells (5×10^4 cells mL^{-1}). Viral titre was determined by flow cytometric analysis of the percentage of GFP-expressing cells 6 days after of infection. The capture efficiency is expressed as the percentage of decrease in titre of the viral supernatant. In addition, The Streptavidin MagneSpheres[®] were washed with HBSS, resuspended in 10 μL of RPMI 1640,

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and then assayed by infection of K562 cells (4×10^5 cells mL⁻¹). The titre of adsorbed viruses on the Streptavidin MagneSpheres[®] before elution was determined. For the elution test, the Streptavidin MagneSpheres[®] were resuspended in 1 mL of 0.6, 1 and 3 mM biotin (dissolved in pH 7.4 D-PBS) for 2 h at room temperature using a rotary mixer. The titre of remaining viruses on the Streptavidin MagneSpheres[®] was determined similarly by infection of K562 cells, and then the elution efficiency was calculated. The error bars represent standard deviations of triplicate samples.

Fig. 7. Column-based purification of desthiobiotinylated lentiviral vectors produced by DBL cells contacted with 2 mM 7-DAPA for 96 h. (A) Pierce monomeric avidin coated columns (2 mL) were loaded with 12 mL of crude 7-DAPA-containing lentiviral feedstocks by gravity flow, washed with 8 mL of PBS buffer, and eluted with 22 mL of 2 mM biotin. Column fractions of breakthrough, wash and elution were analysed by infection of K562 cells (4×10^5 cells mL⁻¹) and the titre of each fraction was determined by flow cytometric analysis of the percentage of GFP-expressing cells after 6 days of infection. (B) Elution profile of these lentiviral vectors in the monomeric avidin coated column at a flow rate of 0.4 mL min⁻¹. The error bars represent standard deviations of triplicate samples.

DETAILED DESCRIPTION

VIRAL VECTOR

In a first embodiment, the present invention provides a producer cell for the production of a viral vector.

Viral vector or viral delivery systems include, for example, adenoviral vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, retroviral vectors (including lentiviral vectors) and baculoviral vectors.

The vector system may be a retroviral vector system.

The concept of using viral vectors for gene therapy is well known (Verma and Somia (1997) Nature 389:239-242).

5 There are many retroviruses. For the present application, the term “retrovirus” includes: murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney
10 murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV) and all other retroviridae including lentiviruses.

Lentiviruses also belong to the retrovirus family, but they can infect both dividing
15 and non-dividing cells (Lewis et al (1992) EMBO J. 3053-3058).

The lentivirus group can be split into “primate” and “non-primate”. Examples of primate lentiviruses include the human immunodeficiency virus (HIV), the causative agent of human acquired immunodeficiency syndrome (AIDS), and the
20 simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype “slow virus” visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

25

Details on the genomic structure of some lentiviruses may be found in the art. By way of example, details on HIV and EIAV may be found from the NCBI Genbank database (i.e. Genome Accession Nos. AF033819 and AF033820 respectively). Details of HIV variants may also be found at <http://hiv-web.lanl.gov>. Details of
30 EIAV variants may be found through <http://www.ncbi.nlm.nih.gov>.

During the process of infection, on entry into the susceptible host cell, the retroviral RNA genome is copied to DNA by the virally encoded reverse transcriptase which is carried inside the parent virus. This DNA is transported to the host cell nucleus where it subsequently integrates into the host genome. At this stage, it is typically referred to as the provirus. The provirus is stable in the host chromosome during cell division and is transcribed like other cellular genes. The provirus encodes the proteins and other factors required to make more virus, which, for a wild-type replication competent virus can leave the cell by a process sometimes called “budding”.

In a simple retrovirus, such as a type C retrovirus or oncoretrovirus, each retroviral genome comprises genes called *gag*, *pol* and *env* which code for virion proteins and enzymes. The gene *gag* encodes the internal structural protein of the virus. Gag protein is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The *pol* gene encodes the reverse transcriptase (RT), which contains DNA polymerase, associated RNase H and integrase (IN), which mediate replication of the genome. The *env* gene encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction leads ultimately to infection by fusion of the viral membrane with the cell membrane.

As used herein the term “viral vector”, when referring to a viral vector system also includes a vector particle capable of transducing a recipient cell with a nucleotide of interest (NOI).

A vector particle includes the following components: a vector genome, which may contain one or more NOIs, a nucleocapsid encapsidating the nucleic acid, and a membrane surrounding the nucleocapsid.

The term “nucleocapsid” refers to at least the group specific viral core proteins (*gag*) and the viral polymerase (*pol*) of a retrovirus genome. These proteins

encapsidate the packagable sequences and are themselves further surrounded by a membrane containing an envelope glycoprotein.

Once within the target cell, the RNA genome from a retroviral vector particle is
5 reverse transcribed into DNA and integrated into the DNA of the recipient cell.

The term "vector genome" refers to both to the RNA construct present in the retroviral vector particle and the integrated DNA construct. The term also embraces a separate or isolated DNA construct capable of encoding such an RNA genome. A
10 retroviral or lentiviral genome should comprise at least one component part derivable from a retrovirus or a lentivirus. The term "derivable" is used in its normal sense as meaning a nucleotide sequence or a part thereof which need not necessarily be obtained from a virus such as a lentivirus but instead could be derived therefrom. By way of example, the sequence may be prepared synthetically
15 or by use of recombinant DNA techniques.

PRODUCER CELLS

The separate expression of the components required to produce a retroviral vector
20 particle on separate DNA sequences cointroduced into the same cell will yield retroviral particles. This cell is referred to as the producer cell.

There are two common procedures for generating producer cells. In one, the sequences encoding retroviral Gag, Pol and Env proteins are introduced into the cell
25 and stably integrated into the cell genome; a stable cell line is produced which is referred to as the packaging cell line. The packaging cell line produces the proteins required for packaging retroviral RNA but it cannot bring about encapsidation due to the lack of a *psi* region. However, when a vector genome (having a *psi* region) is introduced into the packaging cell line, the helper proteins can package the *psi*-
30 positive recombinant vector RNA to produce the recombinant virus stock. This can be used to transduce the NOI into recipient cells. The recombinant virus whose genome lacks all genes required to make viral proteins can infect only once and

cannot propagate. Hence, the NOI is introduced into the host cell genome without the generation of potentially harmful retrovirus.

The producer cell of the present invention may be a packaging cell. The packaging
5 cell line may be transduced with a viral vector system comprising the genome or transfected with a plasmid carrying a DNA construct capable of encoding the RNA genome. The present invention also provides a kit for producing a retroviral vector system which comprises a packaging cell and a retroviral vector genome.

10 The second approach is to introduce the three different DNA sequences that are required to produce a retroviral vector particle (i.e. the *env* coding sequences, the *gag-pol* coding sequence and the defective retroviral genome containing one or more NOIs) into the cell at the same time by transient transfection and the procedure is referred to as transient triple transfection. WO 94/29438 describes the production of
15 producer cells *in vitro* using this multiple DNA transient transfection method. WO 97/27310 describes a set of DNA sequences for creating retroviral producer cells either *in vivo* or *in vitro* for re-implantation.

The components of the viral system which are required to complement the vector
20 genome may be present on one or more “producer plasmids” for transfecting into cells.

The present invention also provides a kit for producing a producer cell of the invention, comprising

- 25 (i) a viral vector genome which is incapable of encoding one or more proteins which are required to produce a vector particle;
- (ii) one or more producer plasmid(s) capable of encoding the protein which is not encoded by (i); and optionally
- (iii) a cell suitable for conversion into a producer cell.

30 The viral vector genome may be incapable of encoding the proteins *gag*, *pol* and *env*. The kit may thus comprise one or more producer plasmids encoding *env*, *gag*

and pol, for example, one producer plasmid encoding env and one encoding gag-pol.

By using producer/packaging cell lines, it is possible to propagate and isolate
5 quantities of retroviral vector particles (e.g. to prepare suitable titres of the retroviral vector particles) for subsequent transduction of a site of interest.

Producer cells/packaging cells can be of any suitable cell type. Producer cells are generally mammalian cells but can be, for example, insect cells.

10

As used herein, the term “producer cell” refers to a cell which contains all the elements necessary for production of retroviral vector particles.

As used herein, the term “packaging cell” refers to a cell which contains those
15 elements necessary for production of infectious recombinant virus which are lacking in the RNA genome. Typically, such packaging cells contain one or more producer plasmids which are capable of expressing viral structural proteins (such as *gag-pol* and *env*, which may be codon optimised) but they do not contain a packaging signal.

20

The term “packaging signal” which is referred to interchangeably as “packaging sequence” or “*psi*” is used in reference to the non-coding, *cis*-acting sequence required for encapsidation of retroviral RNA strands during viral particle formation. In HIV-1, this sequence has been mapped to loci extending from upstream of the
25 major splice donor site (SD) to at least the *gag* start codon.

Packaging cell lines may be readily prepared (see also WO 92/05266), and utilised to create producer cell lines for the production of retroviral vector particles.

30 The packaging cell lines are useful for providing the gene products necessary to encapsidate and provide a membrane protein for a high titre vector particle production. The packaging cell may be a cell cultured *in vitro* such as a tissue

culture cell line. Suitable cell lines include but are not limited to mammalian cells such as murine fibroblast derived cell lines or human cell lines. The packaging cell line may be a human cell line, such as for example: HEK293, 293-T, TE671, HT1080.

5

PROTEIN OF INTEREST

In a second embodiment, the present invention provides a cell for the production of a protein of interest.

10

The protein of interest (POI) may be any protein which may be produced by the cell. It may, for example, be a therapeutically or diagnostically useful protein.

15

For example the POI may be one of the following: cytokine, chemokine, hormone, antibody, anti-oxidant molecule, engineered immunoglobulin-like molecule, a single chain antibody, fusion protein, enzyme, immune co-stimulatory molecule, immunomodulatory molecule, a transdominant negative mutant of a target protein, a toxin, painal toxin, an antigen, a tumour suppresser protein, growth factor, vasoactive protein or peptide, anti-viral protein, receptor protein or an ion channel.

20

The protein of interest may be made by introduction of the gene encoding the POI into the cell. The gene encoding the POI may be introduced, for example, as part of an expression vector, such as a plasmid.

25

The expression vector may also comprise a gene encoding a biotinylation competent entity, such as an acceptor sequence (see below). The POI may be expressed by the cell as a fusion protein with the acceptor sequence.

30

The cell may be any cell capable of expressing the POI and a biotin/desthiobiotin-tagged POI in the presence of the relevant substrate, such as 7-DAPA or 7-KAPA.

The cell may, for example, be a bacterial, yeast or mammalian cell.

BIOTIN BIOSYNTHESIS

Biotin is a vitamin that functions as an enzyme co-factor in cellular metabolism.

5 Biosynthesis of biotin from pimeloyl-CoA and Ala occurs through four reactions that result in the sequential production of:

1. 7-keto-8-amino perlargonic acid (7-KAPA)
2. 7,8-diaminoperlargonic acid (7-DAPA)
- 10 3. desthiobiotin
4. biotin

The biosynthetic pathway from 2 to 4 is shown in Figure 1.

15 The four genes involved in this pathway in some bacteria and plants are shown in the following table:

E. coli gene	Arabidopsis gene	Enzymatic product
<i>bioF</i>	BIO4	KAPA
<i>bioA</i>	BIO1	DAPA
<i>bioD</i>	BIO3	desthiobiotin
<i>bioB</i>	BIO2	biotin

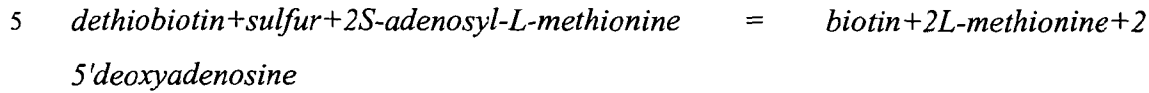
20 In order to synthesise desthiobiotin from 7-DAPA, the cell of the first aspect of the invention may express *bioD*, BIO3 or a homolog or ortholog thereof.

In order to synthesise desthiobiotin from 7-KAPA, the cell of the first aspect of the invention may also express *bioA*, BIO1 or a homolog or ortholog thereof.

25

In order to synthesise biotin from 7-DAPA or 7-KAPA, the cell of the first aspect of the invention may also express *bioB*, BIO2 or a homolog or ortholog thereof.

BioB, BIO2 or a homologs/orthologs thereof catalyse the following reaction:



Sulphur and/or S-adenosyl-L-methionine (SAM) may be provided directly or indirectly to the cell. Alternatively one or both cofactors may be present in situ; for example SAM is synthesised by human cells from L-methionine, and sulphur may be produced via the breakdown of methionine or cysteine.

The cell may express a fusion protein, such as bioD-bioA, BIO3-BIO1, bioD-bioA-bioB or BIO3-BIO1-BIO2 (the proteins being in any order). BIO3-BIO1 fusion
15 proteins have been found to exist naturally in plants (Muralla *et al* (2008) Plant
physiol 146:60-73)

The amino acid sequence for E. coli BioD is shown as SEQ ID No. 1.

20 The amino acid sequence for E. coli BioA is shown in SEQ ID No. 2.

The amino acid sequence for *E. coli* BioB is shown as SEQ ID No. 3.

The amino acid sequence for BIO3-BIO1 is shown as SEQ ID No 4.

The amino acid sequence for BIO2 is shown as SEQ ID No. 5.

The term "ortholog" as used herein means an enzyme having the same function which is derivable from a different species.

The term “homolog” is used to mean a polypeptide having a primary amino acid sequence which differs from the wild-type sequence by one or more amino acid additions, substitutions or deletions but which is capable of the enzymatic activity

characteristic of the wild-type enzyme (e.g. converting 7-DAPA or 7-KAPA into desthiobiotin). The homolog may arise naturally, or may be created artificially (for example by site-directed mutagenesis). The homolog may be at least 75, 85 or 90% identical, for example at least 95 or 98% identical to the subject sequence. The homolog may have 20 mutations or less over the whole wild-type sequence. The homolog may have 10 mutations or less, for example 5 mutations or less over the whole wild-type sequence.

Homology or identity comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % identity between two or more sequences.

% identity may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an “ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % identity when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximise local identity.

However, these more complex methods assign “gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness

between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % identity therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

5

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

10

15

ACCEPTOR SEQUENCE

The viral vector or POI produced by the method of the first aspect of the invention comprises or is associated with a sequence which is capable of being labelled with biotin/desthiobiotin.

20

The acceptor sequence may contain a lysine residue which can form an amide bond with the carboxyl group of desthiobiotin.

With regard to the second embodiment of the first aspect of the invention, the POI may naturally comprise an acceptor sequence, or it may be introduced artificially.

5 In connection with the first embodiment of the first aspect of the invention (production of viral vectors) the acceptor sequence may be expressed at the surface of the vector particle. This may be achieved, for example, by having or introducing an acceptor sequence in a viral surface protein such as a viral envelope protein or fiber capsid protein. A disadvantage with this approach, however, is that, each viral vector type produced by the cell would require modification of the relevant gene,
10 such as the *env* gene.

An alternative strategy is to modify the producer cell, such that all viruses produced by the cell will be associated with an acceptor sequence. This may be achieved, for example by incorporating an acceptor sequence into the sequence of an endogenous
15 or exogenous protein which is expressed at the cell surface which co-packages with the viral vector. Hence, the viral vectors acquire desthiobiotinylated envelope proteins as they bud from the producer cell membrane. The non-viral host-derived protein thus labels the surface of viral vectors produced by the cell, such that they can be captured with avidin.

20

An example of such an exogenous protein, which is expressed at the cell surface and into which an acceptor sequence may be introduced is the low-affinity nerve growth factor receptor (Δ LNGFR). An acceptor peptide may be fused to the extracellular domain of Δ LNGFR.

25

Thus, in connection with the first, third and fourth embodiments, the cell of the first aspect of the invention may comprise a biotin/desthiobiotin acceptor sequence in a cell surface protein. An exogenous cell membrane protein comprising such a sequence may be expressed in the cell such that the cell and/or an enveloped virus
30 budding from the cell, will be tagged with biotin/desthiobiotin.

In connection with the second embodiment of the invention (production of a POI) the acceptor sequence may be part of the POI, either because the POI comprises such a sequence, or because it has been artificially introduced. Alternatively, the acceptor sequence may be directly or indirectly linked to the POI. For example, the acceptor sequence may be co-expressed with the POI as a fusion protein (see, for example, US patent No 5,874,239).

The acceptor sequence may comprise or consist of a 13-amino acid consensus sequence identified by Schatz (1993, Biotechnology 11:1138-1143; US Patent No 5,723,584).

The sequence according to Schatz is summarised in the table below:

L	X	§ (not large hydrophobic)	I	F	E	A	Q	K	I	E	W	R
				L	D	G	M		M	L	Y	H
						S			V	V	V	
						T				Y	F	
										I	L	
											I	

For example, the acceptor sequence may comprise the peptide: LNDIFEAQKIEWH.

The acceptor sequence may comprise the peptide: GGGLNDIFEAQKIEWHE.

The biotin/desthiobiotin acceptor sequence may be, or be part of a protein having a biotinylation site, such as: the tomato cDNA biotin protein sequence; the alpha subunit of *Klebsiella pneumoniae* oxalacetate decarboxylase (US Patent No 5,252,466); pyruvate carboxylase; propionyl-CoA carboxylase; acetyl CoA carboxylase, methylcrotonyl-CoA carboxylase; and a *P. shermanii* transcarboxylase domain (PSTCD) peptide (US patent application No US 2002/0142355).

LIGASE ENZYME

The cell may comprise an enzyme capable of biotinylating/desthiobiotinylating an acceptor sequence. For example, the cell may express a heterologous ligase such as
5 E. coli biotin ligase (BirA) or an ortholog or homolog thereof. The sequence encoding a heterologous ligase may comprise elements which aid translation of the enzyme the endoplasmic reticulum lumen (such as the mammalian ER signal peptide) and/or which reduce leakage into the culture supernatant (such as the
10 KDEL tetrapeptide eukaryotic ER retention signal).

It has been shown that BirA can cause the *in vitro* desthiobiotinylation of recombinant proteins carrying a biotinylation tag (Wu and Wong (2004) Anal. Biochem. 340-348).

15

The amino acid sequence for BirA is shown as SEQ ID No. 6.

Alternatively the cell may have an endogenous pathway for biotinylating/desthiobiotinylating proteins (US patent application No US
20 2002/0142355 refers to an endogenous biotinylation pathway).

DUAL TAGGING

The incorporation of a polyhistidine tag (e.g. a His₆ tag) to viral vectors or POIs
25 means that the vectors/POIs can be subjected to immobilised metal affinity chromatography (IMAC) using commercially available NTA columns.

If the vectors/POIs produced by the method of the invention are also His₆-tagged, the resultant desthiobiotin- and His₆- dual tagged vectors/POIs can be sequentially
30 processed by IMAC and avidin, thus extending the range of potential affinity mediated purification matrices.

His₆-tagging may be achieved by incorporating a His₆ sequence, in the same way as a desthiobiotin acceptor sequence may be incorporated. For example in producer cells, a linker coding for His₆ sequences may be inserted into the Δ LNGFR coding sequence.

5

A procedure for separating histidine-tagged lentiviral vectors from culture supernatant using immobilised metal affinity chromatography has been described (Cheeks *et al* (2009) J. Chromatography 1216:2705-2711; Ye *et al* (2004) J Virol 78:9820-9827; Yu and Schaffer (2006) J Virol 80:3285-92)

10

METHOD FOR PRODUCING A CELL

In a second aspect, the present invention provides a method for producing a cell according to the first aspect of the invention, which comprises the step of
15 introducing a nucleotide sequence which encodes an enzyme which catalyses the conversion of 7,8-Diaminoperlargonate (7-DAPA) or (7-KAPA) into biotin/desthiobiotin into the cell.

The enzyme may be able to synthesise desthiobiotin from 7-DAPA, such as bioD,
20 BIO3 or a homolog or ortholog thereof.

In order to synthesise desthiobiotin from 7-KAPA, a nucleotide sequence encoding an enzyme which is able to synthesise 7-DAPA from 7-KAPA may also be introduced, such as bioA, BIO1 or a homolog or ortholog thereof.

25

In order to synthesise biotin, a nucleotide sequence encoding an enzyme which is able to synthesise biotin from desthiobiotin may also be introduced, such as bioB, BIO2 or a homolog or ortholog thereof

The nucleotide may encode a fusion protein, such as bioD-bioA, BIO3-BIO1, bioD-bioA-bioB or BIO3-BIO1-BIO2. The proteins may be in any order within the fusion protein.

- 5 The method may also comprise the step of introducing a nucleotide sequence capable of encoding a biotin ligase enzyme to the cell. The ligase enzyme may, for example, be BirA or an ortholog or homolog thereof.

10 The method may also comprise the step of introduction of a biotin/desthiobiotin acceptor sequence to the cell.

In connection with production of viral vectors, viral clearance or the production of labelled cells, a sequence encoding a cell-membrane protein comprising, or fused to, a biotin/desthiobiotin acceptor sequence may be introduced to the cell. The cell-
15 membrane protein may, for example be Δ LNGFR.

METHOD FOR PRODUCING VIRAL VECTOR

20 In a third aspect, the present invention provides a method for producing a biotin/desthiobiotin-tagged viral vector which comprises the step of production of the viral vector using a producer cell according to the first aspect of the invention in the presence of 7-DAPA or 7-KAPA.

The method may comprise the step of:

- 25 (i) introducing the vector genome to the cell
(ii) introducing any genes encoding any structural proteins not provided by the cell (such as *gag*, *pol* and/or *env*)
(iii) allowing vector production to occur in the presence of 7-DAPA or 7-KAPA; and
30 (iv) harvesting viral vectors.

7-DAPA or 7-KAPA may be present in the culture medium. The culture medium may comprise essentially no free biotin.

METHOD FOR PRODUCING A POI

5

The present invention also provides a method for producing a biotin/desthiobiotin-tagged protein of interest (POI) which comprises the step of expression of the POI from a cell according to the first aspect of the invention in the presence of 7-DAPA or 7-KAPA.

10

The method may comprise the following steps:

- (i) introducing a nucleotide sequence encoding a POI to the cell; and
- (ii) allowing POI production to occur in the presence of 7-DAPA or 7-KAPA.

15

7-DAPA or 7-KAPA may be present in the culture medium. The culture medium may comprise essentially no free biotin.

20

The nucleotide sequence may also encode a biotin/desthiobiotin acceptor sequence, for example, within or adjacent to the POI-encoding sequence, so that they are co-expressed.

PURIFICATION METHODS

25

In a fourth aspect, the present invention provides a method for purification of a viral vector/protein of interest (POI), which comprises the following steps:

- (i) producing a viral vector or a POI by a method according to the second aspect;
- (ii) avidin-based capture of the vector/POI; and
- (iii) elution of the captured vector/POI.

30

Monomeric avidin has a lower affinity binding to biotin than streptavidin, so enables the captures vector/POI to be eluted more readily.

Biotin/desthiobiotin-tagged viral vectors or POIs may be captured by methods
5 known in the art, such as by centrifugation, affinity chromatography, or particle-based capture, for example using paramagnetic particles (Chan *et al* (2005) J. Virol. 79:13190-13194; Hughes *et al* (2001) Mol. Ther. 3:623-630).

In view of the fact that 7-DAPA and 7-KAPA have little or no affinity for avidin,
10 there is no need for this material to be removed prior to the avidin-based capture step. Hence the method of the invention may lack any low specificity purification processes such as centrifugation, precipitation, filtration or ion-exchange chromatography in order to remove contaminants from the supernatant.

15 The association between desthiobiotin and avidin is reversible, so captured desthiobiotin-labelled viral vectors or POIs may be eluted by gentle procedures.

The association between biotin and avidin is virtually irreversible, which may be useful for e.g. viral clearance and uses of labelled cells where a strong association
20 with avidin is desired.

PLASMIDS AND KITS

The fifth aspect of the invention relates to plasmids and kits for use in connection
25 with the invention. The plasmids may be for use in generating a cell according to the first aspect of the invention.

The term "plasmid" is used to indicate any vector suitable for transferring DNA into a cell. It includes other vectors, such as viral vectors, naked DNA etc.

30 The plasmid may comprise a nucleotide sequence encoding one or more of the following:

(i) an enzyme capable of synthesising desthiobiotin from 7-DAPA, such as bioD, BIO3 or a homolog or ortholog thereof;

(ii) an enzyme capable of converting 7-KAPA to 7-DAPA, such as bioA, BIO1 or a homolog or ortholog thereof;

5 (iii) an enzyme capable of converting desthiobiotin to biotin, such as bioB, BIO2 or a homolog or ortholog thereof; and

(iv) a ligase enzyme capable of covalently coupling desthiobiotin to an acceptor site, such as BirA or a homolog or ortholog thereof.

10 In connection with the first embodiment of the invention (production of viral vectors) one or more of the following may also be provided:

(i) a plasmid comprising the viral genome;

(ii) one or more plasmids encoding proteins required by the producer cell in order to produce a viral particle, such as Gag, Pol and/or Env; and

15 (iii) a plasmid encoding a biotin/desthiobiotin acceptor sequence, optionally as part of or fused to an exogenous cell membrane protein; and

(iv) a plasmid encoding a His₆ sequence, optionally as part of or fused to an exogenous cell membrane protein.

20 In connection with the second embodiment of the invention (production of a POI) one or more of the following may also be provided:

(i) a plasmid comprising a nucleotide sequence encoding the POI optionally joined to a nucleotide sequence encoding a biotin/desthiobiotin acceptor and/or a nucleotide sequence encoding a His₆ sequence.

25

Two or more of the above-mentioned plasmids may be provided together in the form of a kit, suitable for producing a cell, tagged viral vector, or tagged POI according to the present invention.

30 The kit may also comprise 7-KAPA, 7-DAPA or a compound suitable for converting into 7-KAPA or 7-DAPA for use in connection with the present invention.

The kit may also comprise one of both of the co-factors sulphur and S-adenosyl-L-methionine for an enzyme to convert desthiobiotin to biotin, or entities capable of producing such co-factors *in situ*.

5

VIRAL CLEARANCE

The invention also provides a method for the purification of an entity of interest (EOI) which comprises the following steps:

10 (i) production of the EOI using a cell according to the third embodiment of the invention, in the presence of 7-DAPA or 7-KAPA; and

(ii) avidin-based removal of the biotin/desthiobiotin tagged adventitious agent.

15 The EOI may be any entity produced by the cell which is untagged by the biotin/desthiobiotin labelling system. The EOI may, for example, be a peptide, polypeptide or protein. The EOI may be an antibody, such as a monoclonal antibody, or a fragment or a fusion thereof. Antibody fragments include Fab, scFv, monobodies, diabodies and triabodies, minibodies and single domain antibodies.

20

The adventitious agent may be any agent produced by the cell which is tagged by the biotin/desthiobiotin labelling system. The adventitious agent may, for example, be an enveloped virus. The agent may become associated with a membrane protein from the producer cell which comprises a biotin/desthiobiotin acceptor sequence
25 such that the agent is labelled and thus distinguishable from the EOI.

The present invention also provides a method for the removal of a tagged adventitious agent from a preparation of an EOI, which comprises the step of avidin/streptavidin-based capture of the tagged adventitious agent.

30

The adventitious agent may be removed by for example passage of the product stream through an avidin/streptavidin functionalised filter, such as a short chromatography column or a membrane.

- 5 The advantage of using a biotin/desthiobiotin-labelling approach for viral clearance is that all enveloped viruses budding from the cell which retain the correct portions of the outer membrane of the cell will be tagged, so it is possible to remove them all without further identification.
- 10 The viral clearance method of the present invention may be used in conjunction with one or more other method(s), such as virus retentive filtration, anion-exchange column chromatography, ultra violet-c (UVc) virus inactivation and membrane chromatography.
- 15 The biotin/desthiobiotin labelling system a cheap, simple to conduct and is "orthogonal" (works by a different mechanism) to conventional virus removal mechanisms. Making it an attractive option/addition for use in the process.

20

LABELLED CELLS

The present invention also produces a method for producing a biotin- or desthiobiotin-coated cell, which comprises the step of culturing a cell according to
25 the fourth embodiment of the invention in the presence of 7-DAPA or 7-KAPA, and a biotin- or desthiobiotin-coated cell produced by such a method.

The generation of desthiobiotin and/or biotin coated/modified cells allows the use of avidin/streptavidin mediated manipulation of these cells, either in a reversible
30 (with desthiobiotin, due to its lower affinity for avidin/streptavidin) or virtually irreversible manner (with biotin due to its higher affinity for avidin/streptavidin).

Such modified cells can then be used for a variety of *in vitro* manipulations, e.g. attachment to avidin coated scaffolds to generate specific tissue shapes, or similarly for arrangement of different cell types in specific juxtaposition to each other.

5 For example, avidin/streptavidin paramagnetic beads may be attached to the modified cells, thus allowing the use of magnets for the attraction of the modified cells. In the *in vitro* applications, this could be use for purification and/or various forms of cell patterning/organisation. In the *in vivo* applications, external or even internally placed magnets (e.g. magnetised stents) may be used to attract and retain
10 the modified cells in a particular location. In addition to paramagnetic beads the biotin/avidin affinity may similarly be used for the attachment of fluorescent or other labels/moieties.

The genetic modification mediated generation of the desthiobiotin/biotin coated
15 cells allows the continued biotin/desthiobiotin modification of the daughter cells, unlike chemically modified cells which rapidly lose biotin in the daughter cells.

Genetic modification has the added advantage of allowing the use of the precursor molecules (DAPA/KAPA), thus preventing contamination of the microenvironment
20 with free biotin.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

25

EXAMPLES

Materials

30 Streptavidin Magnespheres[®] paramagnetic particles (1 mg mL⁻¹) were supplied by Promega (Southampton, UK). Barium hydroxide monohydrate was obtained from Fisher (Loughborough, UK). RPMI-1640 medium (without L-glutamine),

Dulbecco's modified Eagle medium (DMEM), CellMask™ plasma membrane stain, NuPAGE® Novex® Bis-Tris 4-12% Mini Gels, Novex® MES SDS Running Buffer (20×) and Novex® LDS sample buffer (4×) were purchased from Invitrogen (Paisley, UK). Pierce SilverSnap® Stain Kit and monomeric avidin coated Pierce columns were purchased from Thermo Scientific (Rockford, USA). Desthiobiotin, foetal bovine serum (FBS), dialysed FBS (10,000 Da molecular weight cut-off), Dulbecco's phosphate buffered saline (D-PBS), penicillin-streptomycin solution (10,000 U mL⁻¹ penicillin and 10 mg mL⁻¹ streptomycin), L-glutamine solution (200 mM), puromycin, blasticidin, hygromycin, avidin-FITC, 4-hydroxyazobenzene-2-carboxylic acid (HABA)-avidin reagent, trypsin-EDTA solution (0.5 g L⁻¹ porcine trypsin and 0.2 g L⁻¹ EDTA·4Na) and Hanks' balanced salt solution (HBSS) were obtained from Sigma-Aldrich (Dorset, UK).

Synthesis of 7-DAPA

7-DAPA was chemically synthesised according to the procedure illustrated in Fig. 2. A 20-mL Biotage Microwave Vial (Biotage, Sweden) was added with 160 mg of desthiobiotin, 16 mL of deionised water, 3.53 g of barium hydroxide monohydrate as a catalyst and a magnetic stir bar, and then sealed. The reaction proceeded at 158°C in an Initiator Microwave Synthesiser (Biotage, Sweden) for 7.5 h. The sample was then centrifuged twice to remove insoluble impurities. The supernatant was saturated with carbon dioxide and the precipitate was removed by centrifugation. The resulting supernatant was acidified with 1 M H₂SO₄ (pH ~6.5) to remove residual barium ions, and then lyophilised to fine white powder (yield = 62%).

Structural characterisation

¹³C-NMR spectra of 7-DAPA (in D₂O) and desthiobiotin (in d₆-DMSO) were obtained on a Bruker Advance 500 MHz spectrometer (Bruker Biospin GmbH, Germany) at room temperature. High-resolution mass spectrometry (HRMS) was carried out using a Micromass Quadrupole-Time of Flight spectrometer. ¹³C-NMR (500 MHz, D₂O) for 7-DAPA: δ 183.9, 53.7, 49.0, 37.3, 29.9, 28.1, 25.3, 24.4, 13.6 ppm; HRMS (*m/z*) for [C₉H₂₁N₂O₂]⁺, calculated: 189.1603, found: 189.1610.

Cell culture

Human 293T based BL15 packaging cells (Nesbeth *et al.* 2006; as above) were grown in DMEM supplemented with 10% (v/v) biotin-free FBS, 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, 2.5 µg mL⁻¹ blasticidin and 2.5 µg mL⁻¹ puromycin. In this cell line the extracellular domain of a fragment of the low-affinity nerve growth factor receptor (LNGFR) has been fused to a biotin acceptor peptide (BAP). The expression of LNGFR-BAP with the bacterial birA gene in these cells has enabled the endogenous metabolic biotinylation of a specific lysine residue in the BAP region. The biotin is thus transported to the cell surface as a membrane anchored affinity moiety and incorporated onto the membrane of the lentiviral particles produced by these cells. The DBL packaging cell line has been developed by cloning and expressing synthase bioD alongside birA and LNGFR-BAP in BL15 cells. DBL cells were grown in DMEM supplemented with 10% (v/v) biotin-free FBS, 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin, 2.5 µg mL⁻¹ blasticidin, 2.5 µg mL⁻¹ puromycin and 200 U mL⁻¹ hygromycin. K562 human immortalised myelogenous leukaemia cells were maintained in RPMI-1640 medium and TE671 human rhabdomyosarcoma cells were maintained in DMEM. Both of these sets of medium were supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. The four types of cells were split three times per week. Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Laser scanning confocal microscopy

To visualise the cell surface desthiobiotinylation, 4×10^5 DBL cells were cultured on a 35-mm glass-bottom culture dish (MatTek, USA) with 2 mM 7-DAPA for 96 h. The spent medium was removed gently and the cells were incubated in 1 mL of fresh complete DMEM containing 5 µg mL⁻¹ CellMask™ plasma membrane stain at 37°C for 5 min. The stain was removed and the cells were washed gently three times with D-PBS. 2 mL of complete DMEM containing 80 µg mL⁻¹ avidin-FITC was then added to the cells at 4°C for 30 min. The FITC was aspirated and the cells were washed with D-PBS twice. An SP5 laser scanning confocal microscope

(Leica, Germany) was used to image the desthiobiotinylated DBL cells. The avidin-FITC and CellMask™ plasma membrane stain were excited using laser lines of 488 and 543 nm respectively.

5 *HABA-avidin assay*

The extent of 7-DAPA mediated desthiobiotinylation on the DBL cells was determined by using the HABA-avidin assay (Tsai et al Biomaterials 2005, 26, 3141-3151; Ho et al Biomaterials 2009, doi:10.1016/j.biomaterials.2009.08.023, In press). The DBL cells were cultured with 7-DAPA at the described concentration
10 for the indicated time periods. The spent medium was then removed and the cells were rinsed with D-PBS. The absorbance of fresh HABA-avidin solution, which contains 0.3 mM HABA, 0.45 mg mL⁻¹ avidin, 0.01 M HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], a buffer with pKa = 7.5), 0.3 M NaCl, 0.01 M MgCl₂ and 0.02% sodium azide, was first measured at 500 nm and
15 2 mL of the solution was added to the adherent DBL cells in a culture flask and incubated for 5 min. The HABA-avidin solution was then removed and its absorbance was measured at 500 nm. The change in absorbance of the HABA-avidin solution and the relevant cell counts were used to calculate the average number of surface desthiobiotin per cell. For the experimental control, DBL cells
20 (cultured in 7-DAPA free medium) were incubated with HABA-avidin solution.

Desthiobiotinylation of lentiviral vectors

Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped lentiviral vectors, which were produced from the 7-DAPA treated DBL packaging cells, acquired
25 desthiobiotinylated envelope proteins as they budded from the DBL cell membrane. Briefly, the DBL cells were trypsinised and plated at 1×10^6 cells per 90 mm Petri-dish in 10 mL of complete DMEM containing 7-DAPA at the described concentration. After 72 h the cells cultured in 7-DAPA-containing medium were transiently transfected with a combination of green fluorescence protein (LV.gfp
30 (Chan et al 2005, as above) lentivirus (vector, 10.0 µg per dish), delta 8.91 (helper, 7.0 µg per dish, Zufferey et al 1997) and VSV-G (envelope, 3.5 µg per dish) plasmids by calcium phosphate transfection. After 48 h the lentiviral supernatant

was harvested and filtered with 0.45 µm filter for processing and infection of target cells.

Determination of viral titre

5 4×10^5 suspension K562 cells were seeded into each well of a 24-well plate containing complete RPMI-1640 medium (1 mL per well containing $4.4 \mu\text{g mL}^{-1}$ polybrene). The plate was placed into a 37°C incubator for 1-2 h. Preparations of freely suspended or Streptavidin Magnespheres® bound viruses were titred as described previously (Nesbeth *et al.* 2006, as above). Briefly, samples were diluted
10 serially 1:10 in complete RPMI-1640 medium and 100 µL was added to triplicate wells with mixing. After 48 h the suspension K562 cells were split by replacing 900 mL of cell suspension with the same volume of fresh complete RPMI-1640 medium. The plate was returned to culture for a further 96 h after which the titre was determined by analysis of the percentage of GFP-expressing cells on a BD
15 FACScan flow cytometer (BD Bioscience, USA). The titre (mL^{-1}) was calculated as the fraction of gfp expressing cells multiplied by the initial target cell number multiplied by the dilution factor. In some case, adherent TE671 cells were chosen as target cells with a seeding density at 5×10^4 cells per well.

20 *Batch capture and elution of lentiviral vectors using Streptavidin Magnespheres®*
Streptavidin Magnespheres® were supplied sterile and prepared by washing three times in HBSS. 100 µL of these streptavidin coated paramagnetic particles were added to 3 mL of the harvested 7-DAPA-containing lentiviral supernatant and mixed at room temperature for 2 h using a rotary mixer. The paramagnetic
25 particles were then separated by a Dynal Magnetic Separator (Invitrogen, UK) and the titres of the viral supernatants before and after the contact period and that of the adsorbed viruses on the Streptavidin Magnespheres® were determined. For the elution study, the desthiobiotinylated lentiviral vectors were desorbed from the Streptavidin Magnespheres® using 0.6, 1 and 3 mM D-biotin (dissolved in pH 7.4
30 D-PBS) respectively. After the lentivirus-adsorbed paramagnetic particles were washed with HBSS twice, 1 mL of D-biotin solution was added to the adsorbent sample and mixed for 2 h at room temperature using a rotary mixer. The titres of

the eluted viruses and the remaining viruses on the Streptavidin Magnespheres[®] were determined.

Column-based purification of lentiviral vectors

5 Monomeric avidin coated Pierce columns (2 mL) were selected to test the affinity column-based recovery of desthiobiotin-tagged lentiviral vectors. The columns were loaded with crude 7-DAPA-containing lentiviral feedstocks by gravity flow, washed with PBS buffer and eluted with 2 mM biotin. Column breakthrough, wash and elution fractions were collected and analysed by flow cytometric analysis of the
10 percentage of GFP-expressing cells after 6 days of infection of target cells.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Samples (5 µL) were heated at 90°C for 30 min using a Stuart block heater (Bibby Scientific, UK) to inactivate viruses. These samples were analysed by SDS-PAGE
15 using NuPAGE[®] Novex[®] Bis-Tris 4-12% Mini Gels and XCell Mini-Cell according to the manufacturer's instructions (Invitrogen, UK). The gel was stained with Pierce SilverSnap[®] Stain Kit according to the manufacturer's instructions (Thermo Scientific, USA) and then photographed on a MEDALight light panel (Morco, UK) using a Cannon EOS 400D Digital SLR Camera (Japan).

20

Example 1 - Synthesis and characterisation of 7-DAPA

The synthesis of Hoffmann *et al* (The Journal of Biological Chemistry 1941, 141, 207-214) was modified to make 7-DAPA from desthiobiotin in a microwave
25 synthesiser. The purified product was analysed by ¹³C-NMR. Compared to the ¹³C-NMR spectrum of the starting desthiobiotin, it is noticeable that the urea carbon group (δ 162.9 ppm) on the cyclic urea derivative had disappeared in the putative 7-DAPA product (Fig. 3). The molecular weight of 7-DAPA was verified by high-resolution mass spectrometry.

30

Example 2 - Desthiobiotinylation of cell surface

The hypothesis that DBL cells with a combination of LNGFR/BAP (acceptor), birA (biotin ligase) and bioD (desthiobiotin synthase) would convert exogenous 7-DAPA into membrane-bound desthiobiotin was tested by confocal microscopy analysis (Fig. 4). Avidin-FITC was able to detect the presence of desthiobiotin (Fig. 4B) and CellMask™ stain was able to locate the position of the cell plasma membrane (Fig. 4C). The overlay of Fig. 4B and C (Fig. 4D) shows that desthiobiotinylation on the surface of DBL cells was successful. No detectable avidin-FITC fluorescence is observed on the parent BL15 cells (Fig. 4A), indicating that DBL cells alone utilised 7-DAPA as a result of BioD expression.

The density of desthiobiotin on the DBL cell surface was quantified by HABA-avidin assay (Fig. 5). Desthiobiotin can displace HABA in the HABA-avidin complex easily due to its higher affinity binding to avidin. The resulting reduction in absorbance of the HABA-avidin solution at 500 nm can be related to the desthiobiotin concentration. Fig. 5A shows that the average number of desthiobiotin molecules on the cell surface was dependent on the amount of 7-DAPA added to the culture. The extent of desthiobiotinylation was only marginally increased at 7-DAPA concentrations lower than 0.5 mM when compared to the control DBL cells without 7-DAPA. The degree of desthiobiotin labelling on the DBL cell membranes however was increased significantly (to 1.2×10^9 desthiobiotin molecules per cell) at 2mM 7-DAPA. Fig. 5B shows that a substantial degree of desthiobiotinylation could be obtained after 24 h. This level increased to a peak at 48 h where further incubation resulted in decreased activity. A control sample in the absence of 7-DAPA was necessary to ascertain the level of background due to non-specific interactions between membrane proteins and HABA (Ho et al, 2009, as above).

Example 3 - Virus capture and elution

DBL cells were cultured continuously in 7-DAPA-containing medium and viral labelling continued from transfection up to the time of viral harvest. Fig. 6A shows that the efficiency of viral capture, indicated by the depletion of VSV-G

pseudotyped lentivirus from the viral supernatant compared with starting viral titre, increased gradually with increasing 7-DAPA to reach 52% at a concentration of 2 mM. It is interesting to note that the variation of the virus capture efficiency is consistent with the extent of desthiobiotinylation previously determined (Fig. 5A).

5 In addition, Fig. 6B shows that the lower extent of desthiobiotinylation observed at 0.5 mM 7-DAPA resulted in the higher elution efficiency of the desthiobiotin-tagged lentiviral vectors. It has been suggested by Williams *et al* (Biotechnology and Bioengineering 2005, 89, 783-787) that the ligand density on the viral surface is a critically important parameter for viral capture and elution, and with DBL cell
10 can be adjusted by varying the degree of labelling of the packaging cells. Fig. 6B shows that over 65% of these adsorbed desthiobiotinylated lentiviral vectors can be eluted by using biotin solution, which is significantly higher than that reported for biotinylated viral vectors (~5%) (Williams *et al* 2005, as above).

15 The capture and elution profiles of the desthiobiotin labelled VSV-G pseudotyped lentiviral vectors were thus used as a basis for the design of a column strategy designed to model processing of low titre lentiviral vector supernatants as they may be harvested from constitutive producer cell lines 12 mL of DBL-derived 7-DAPA-containing crude lentiviral supernatant (2.13×10^6 viral vectors) was applied to a 2-
20 mL Pierce monomeric avidin coated column. As shown in Figure 7A, 89% of lentiviral vectors (1.89×10^6 viral vectors) were captured by the column. The elution efficiency, as determined by the biotin-dependent recovery of captured lentiviruses, reached 77% (1.45×10^6 viral vectors). Analysis of the total lentiviruses eluted compared to the total load indicates an overall yield of purified
25 lentiviral vectors of 68%. For comparison, recovery of active viral vectors post-purification of approximately 30% is taken to be common for currently proposed purification schemes (Rodrigues *et al* J Biotechnol 2007, 127, 520-541). Fig. 7B shows that most viruses can be eluted within 45 min, indicating that 7-DAPA dependent purification of crude feedstocks, where no avidin-binding impurities are
30 required to be removed prior to purification, can achieve very fast and efficient viral recovery. Another recent report of purification of biotinylated-adenovirus by monomeric avidin coated SoftLink™ resin necessitated 4 h initial batch contact

followed by overnight elution in order to improve capture and elution efficiencies (Campos et al Molecular Therapy 2004, 9, 942-954). The purity of the recovered lentiviral vectors was analysed by using the SDS-PAGE analysis. Fig. 8 illustrates that the recovered viruses (Lane 5-8) have no detectable protein impurities. The protein impurities present in the crude virus-containing cell culture supernatant (Lane 2) were found in the supernatant after adsorption (Lane 3) and washing fraction (Lane 4). This indicates an efficient protein clearance and it is noteworthy that since low titre lentiviral vector were applied to the column the individual viral proteins are not visible on this gel.

The development of desthiobiotinylated lentiviruses, which were metabolically produced by DBL packaging cells in contact with 7-DAPA, has two major advantages compared to the metabolic biotinylation technology for scalable virus production: 1) no requirement for pre-treatments to remove free avidin-binding impurities prior to purification; 2) suitability for processes requiring efficient elution of virus from affinity supports. This novel 7-DAPA dependent desthiobiotinylation technology enables affinity-mediated paramagnetic-particle and chromatographic capture and recovery of lentiviral particles with high purity in a highly efficient and fast way. Modifying the number of desthiobiotin molecules on the surface of lentiviral particles by varying the 7-DAPA concentration and incubation time improved the recovery of infectious viral vectors. This provides a potential generic simple affinity recovery process for scalable production of clinical grade lentiviral vectors for gene therapy applications.

Example 4 - Dual polyhistidine/desthiobiotin tagging of lentiviral vectors

Vector Construction

Insertion of His₆ sequences in ΔLNGFRBAP

ΔLNGFRBAP was excised from pLBC3 (Nesbeth 2006, as above) by digestion with BamHI and XhoI and cloned in pBluescript (SK⁺). The single SphI site immediately 3' to the signal peptide of the membrane expressed ΔLNGFRBAP

coding sequence to be used to insert an inframe linker coding for His₆ sequences made by annealing the following oligonucleotides.

5'TGGGCATCACCATCACCATCACGCATG3' and
5'CGTGATGGTGTATGGTGTATGCCACATG3'.

The insertion leaves a single SphI at the 3' end whilst destroying the site at the 5' end. The correct insertion of His₆ sequence was confirmed by bidirectional sequencing of single colony derived plasmid. The resulting "LNGFRBAP.6xHIS" was then ligated into BamHI sites of lentiviral pHR⁻SINctwSVirbl (Chan 2005 as above), upstream of IRES-Blast, confirmed by sequencing and enzyme digestion and designated as pLNGFRBAP.6xHIS.blast.

Modification of cells

293T/BirA/BioD cells as described above were infected with 293T derived VSV-G enveloped lentiviral pLNGFRBAP.6xHIS.blast, selected in 5µg/ml blasticidin S and cryopreserved (293T/BirA/LNGFRBAP.6xHIS.blast). These cells were then incubated for 4 days in 7-DAPA, cultured, expanded, and labelled with 20µg/ml monoclonal mouse anti-polyhistidine (Sigma H1029) and 25µg/ml FITC conjugated rabbit anti mouse immunoglobulins F(ab')₂ secondary and sorted for high cell surface His₆ expression using the Blue (488nm) laser with a 505nm longpass filter and 525/50nm detector. These cells were then expanded, banked and cloned by limiting dilution in the continued presence of puromycin and blasticidin. After 3-4 weeks the expanded clones were screened by Flowcytometry for the dual cell surface His₆/desthiobiotin phenotype.

Antibodies and Immunofluorescence

Cells in 7-DAPA-free FCS (Sigma F-0392), or the indicated amount of biotin were labelled singly or with combinations of with 25µg/ml mouse antihuman NGFR (Becton Dickinson, IgG1 clone C40-1457), 2µg/ml Avidin-FITC (Sigma A2901), 1µg/ml Streptavidin APC (BD 554067) or 20µg/ml mouse anti polyhistidine (Sigma H1029 clone HIS-1) in HBSS+1%FCS for 30 minutes at 4°C. Antibody labelled cells were then incubated in 25µg/ml FITC conjugated rabbit anti mouse

immunoglobulins F(ab')₂ (Dako F0313) in HBSS+1%FCS. After washing and fixing where appropriate (CellFix, Becton Dickinson) the cells were analysed by flow cytometry or sorted.

5 *Lentiviral Vector Production*

Transient transfection of 293T cells produced VSV-G pseudotypes after transfection with pLNGFR.BAP6xHIS.blast at ratios of 3.5:7:10µg using Calcium Phosphate precipitation as previously described (Chan 2005, as above). Lentivirus was harvested 48 hours after transfection and 24 hours after washing and
10 replenishment with sodium butyrate and biotin-free medium and filtered through a 0.45µm filter.

Capture and Concentration of Lentivirus

Lentiviral vectors were captured by either desthiobiotin, NGFR or polyhistidine
15 dependent methods. 1ml (5x10⁸), of streptavidin paramagnetic microparticles (spmps, 1µm in diameter, Promega Z5482), were collected using a Dynal MPC-6 (large volume Magnetic Particle Concentrator).

For desthiobiotin dependent capture -The buffer was aspirated, the spmps resuspended in 1ml of PBS+0.1%BSA and transferred to a 1.5ml microfuge tube.
20 After washing twice with a Dynal MPC-E (small volume Magnetic Particle Concentrator) the spmps were resuspended in 400µl of HBSS+0.1%BSA.

For LNGFR and polyhistidine dependent capture – The spmps were resuspended in 50µl of 2mg/ml biotin conjugated goat polyclonal antibody specific for mouse-IgGFc (Sigma B7401) for 30 minutes at room temperature. After three washes
25 PBS+0.1%BSA the spmps were resuspended in 175µg/ml mouse anti human NGFR antibody or 400µg/ml mouse anti polyhistidine antibody (Sigma H1029 clone HIS-1) in PBS+0.1%BSA. After 30 minutes at room temperature the spmps were washed twice (MPC-E) and resuspended in 400µl of HBSS+0.1%BSA.

After a final MPC-E collection spmps were suspended in 1ml of lentiviral
30 supernatant and incubated at 4°C under agitation (Stuart Scientific roller mixer). After 1.5 hours the mixture was applied to the MPC-6, the supernatant aspirated, and replaced with 1ml of DMEM+10%FCS. After three washes the particles were

resuspended in a final volume of 20 μ l DMEM+10%FCS (50 fold reduction, 1ml to 20 μ l).

Nickel Immobilized Metal Affinity Chromatography

5 GE healthcare (HiTrap) uncharged 5ml IDA Sepharose columns were charged as follows. A Pharmacia peristaltic pump was used to wash the column with 50ml of sterile distilled water; followed by 2.5ml of 0.1M NiSO₄ 1ml/min, followed by 50ml of sterile distilled water and 50ml of 20% ethanol, after which the column could be stored at 4°C until required. Each column could be used 5 times before
10 recharging, performed by stripping off the Nickel with 50ml of sterile distilled water (3ml/min) followed by 30mls of 50mM EDTA after which the column could be recharged. Prior to loading with lentiviral vectors the column was equilibrated (1ml/min) with 50ml of 10mM imidazole containing phosphate buffer pH 7.4 followed by 10ml DMEM+10%FCS with 10mM Imidazole pH 7.4. The virus was
15 harvested, filtered through a 0.45 μ M filter and adjusted to 10mM Imidazole (from 1.5M stock solution pH 7.4) and applied to the column at 1ml/min. Samples were taken of the flow through (BR) to estimate the binding of virus to the column, and subsequent samples taken during the washing steps W1-W3 in 15mM Imidazole in PBS pH 7.4 and the indicated amount of Imidazole in PBS Ph7.4 then used to elute
20 to retained virus (EL1-EL4). VSV-G pseudotyped virus was immediately titre on K562 cells whilst the amphotropic virus was titred on TE671 cells. Prior to purification the VSV-G pseudotyped virus could be stored frozen but the amphotropic virus was always used from a fresh harvest. Estimates of the efficiency of the process were made by comparing the total amount of virus loaded
25 with that passing through the column and that dislodged by the wash step, against the total amount of virus collected in the elution. The estimate of the retention on the column was thus determined by subtracting the total of that which passed through the column prior to elution with the total amount loaded. The elution efficiency was then determined by comparing that eluted with that theoretically
30 removed from the supernatant and retained on the column. This however may also include a component of viral inactivation by the process as a whole and thus elution efficiency must be regarded as a minimum estimate.

Determination of Titre

TE671 cells were plated at 5×10^4 /well in 24 well plates 24 hours prior to infection.

K562 cells were plated in 1ml aliquots at 4×10^5 /ml 3-4 hours prior to infection.

- 5 Serial dilutions of test virus were performed in $4 \mu\text{g/ml}$ polybrene added prior to infection. Titre was determined 6 days after infection using LV.gfp detected by flow cytometry (Chan 2005, Nesbeth 2006).

PAGE and Western Blotting

- 10 Preparation of proteins from the starting material (STG) and elution 1 (EL1) and DMEM+10% FCS were mixed with 1X Laemmli sample buffer and boiled for 10 min. The samples and prestained protein marker (Fermentas, UK) were then loaded onto Precast 4-12% bis tris Nu-PAGE gels (Invitrogen) and separated using a POWERase system (Invitrogen) according to manufacturer's instructions. Some
15 gels for SDS were then stained overnight with comassie blue (0.1% comassie blue 83, 45% methanol and 10% Glacial acetic acid) and destained in 20% Methanol, 7.5% Acetic acid in distilled water. For western blotting the proteins were electrophoretically transferred for 1 hour onto Hybond ECL nitrocellulose membranes (Amersham Bioscience) using Nu-PAGE transfer buffer. The
20 membranes were then blocked for 1 hr in 1XTBS, containing 7.5g of semi-skimmed powder milk, 0.1% tween-20 and 0.05% sodium azide followed by overnight incubation in rabbit anti-HIV-p24 serum (SAIC-Frederick). After three 10 minutes washes in TBS-Tween the membranes were incubated in the appropriate horseradish peroxidase conjugated secondary antibody (DAKO,
25 Denmark) for 1-2 hours. After further washing (3xTBS-Tween) the secondary conjugates were detected by enhanced chemiluminescence (ECL-plus, Amersham Bioscience). The film was developed using photon imaging system SRX-101A (Konica Minolta Medical & Graphic Inc.).

30 **Experimental Design**

A cell line is engineered for the production of lentiviral vectors with high His₆ tag expression combined with streptavidin affinity lentiviral vectors by inserting the

polyhistidine (6x) tag into the previously described pLBC3 vector (Nesbeth 2006, as above). The purpose of the higher His₆ tag is for more efficient IMAC and the streptavidin affinity for subsequent processing if required. This vector is used to modify 293T cells previously modified to express the bacterial biotin ligase BirA and BioD (capable of converting 7-DAPA to desthiobiotin) as described above. The cell line is then sorted, screened and cloned.

The resultant cells are tested by staining with antibody against polyhistidine and their desthiobiotin-dependant avidin-FITC affinity is investigated. Because of the design of the fusion protein, the selected cells should be both desthiobiotin and His₆ tag positive, which may also be confirmed by double labelling with Streptavidin-APC and anti polyhistidine.

To confirm that the dual metal/avidin/streptavidin affinity is passed on to the lentiviral vectors derived from these cells, the cells are transfected to produce VSVG enveloped virus in the presence or absence of 7-DAPA and the capture of these virion compared to that of control 293T cells under the same conditions. Streptavidin paramagnetic particles may be either used as supplied (with affinity for desthiobiotin) or conjugated with antibody to polyhistidine as described.

The capacity for the viral vectors to be captured using IMAC is investigated, using the procedure outlined above, and the viral titres determined in order to calculate the % retention.

The novel dual affinity means that such cells can be used to produce virus with the capability to respond to processing on two quite different affinity columns, IMAC followed by biotin affinity columns, in series, such that purification deficiencies of the first column can be compliment/rectified with the second.

Because the nature of the modification involves neither the envelope gene nor the genetics of the virus, these of modified cells could be used for many envelope viral subsets and pseudotypes. The technology described here could be used for true

“lentiviral packaging cell lines” for dual affinity mediated chromatography for efficient processing of low concentration, alternative pseudotypes.

SEQUENCE LISTING

SEQ ID No. 1 - E. Coli BioD

5 1 mskryfvtgt dtevgktvas callqaakaa gyrtagykp v asgsektpeg lrnsdalalq
 61 mssslqldya tvnpytfaep tsphiisaqe gpiesslvms aglraleqqa dwvlvegagg
 121 wftplsdft fadwvtqeql pvilvvgvkl gcinhamlta qaiqhagltl agwvandvtp
 181 pgkrhaeymt tlrmipapl lgeipwlaen penaatgkyi nlall

10 SEQ ID No. 2 - E. coli BioA

1 mtddldafdq rhiwhpytsm tsplvpypvv saegcelils dgrrlvdgms swwaaihgy
 61 hpqlnaamks qidamshvmf ggithapaie lcrklvamtp qplecvflad sgsvavevam
 121 kmalqywqak gearqrfltf mgyhgdtfg amsvcdpdns mhslwkgylp enlfapapqs
 15 181 rmdgewderd mvfgrlmaa hrheiaavii epivqgaggm rmyhpewlkr irkiedregi
 241 lliadeiatg fgtrgklfac ehaeiapdil clgkaltggt mtlstlttr evaetisnge
 301 agcfmhgptf mgnplacaaa naslaiesg dwqqqvadie vqlreqlapa rdaemvadrv
 361 vlgaigvvet thpvnmaalq kffveqgvwi rpfgklylm ppyiilpqq qrltaavnra
 421 vqdetffcq

20

SEQ ID No. 3 - E. coli BioB

1 mahrpwrls qvtelfekpl ldllfeaqqv hrqhfdprqv qvstlsikt gacpedckyc
 61 pqssryktgl eaerlmeveq vlesarkaka agstrfcmga awknpherm pyleqmvqgv
 25 121 kamgleacmt lgtlsesqaq rlanagldyy nhnldtspef ygniitrtty qerldtlekv
 181 rdagikvcsg givglgetvk dragllqla nlptpesvp inmlvkvkgt pladnddvda
 241 fdfirtiava rimmptsyvr lsagreqmne qtqamcfmag ansifygckl ltpnpeedk
 301 dlqlfrklgl npqqtavlag dneqqqrleq almtptdtey ynnaal

30 SEQ ID No. 4 - Protein encoded by Arabidopsis BIO1-BIO3 bifunctional locus

1 mlvqagghrg qillsrlfqa elaremgyta arfghvmfpe nvyepalkca ellldgvgkg

61 wasrvyfsdn gstaieialk mafkrfcvdh nfceateek hivvkialr gsyhgdltga
 121 meaqapspyt gflqqpwytg rglfldpptv flsngswnis lpesfseiap eygtftrde
 181 ifdksrdast lariysayls khlqehsgvr qsahvgalii epvihgaggm hmvdpfqrq
 241 lvnecmrki pvifdevftg fwrfgvett ellgckpdia cfaklltggm vplavtlatd
 5 301 avfdfsfgds klkallhghs ysahamgeat aakaiqwfk petnhnitsq gktlrelwde
 361 elvqqisshs avqrvvigt lfalelkada snsgyaslya kslimlred giftplgnv
 421 iylmcpets peicrrlltk lykrlegefnr t

SEQ ID No 5 - Arabidopsis BIO2

10

1 mmlvrsvfrs qlrpsvsggl qsascyssl aasaeaerti regprndwsr deiksvydsp
 61 lldllfhgaq vhrhvhnfre vqqctlsik tggcsedcsy cpqssrystg vkaqlmskd
 121 avidaakkak eagstrfcmg aawrdtigrk tnfsqileyi keirgmgmev cctlgmieq
 181 qalelkkagl taynhnldts reypnvitt rsyddrletl shvrdaginv csggiiglge
 15 241 aeedrigllh tlatlpshpe svpinallav kgtpledqkp veiwemirmi gtarivmpka
 301 mvrslagrvr fsmseqalc lagansiftg eklttprnd fdadqlmfkt lglikppsf
 361 seddsesenc ekvasash

SEQ ID No. 6 - E. coli Bir A

20

1 mkdntvplkl iallangefh sgeqlgetlg msraainkhi qtlrdwgvdv ftvpgkgysl
 61 pepiqllnae qilqldggs vavlpvidst nqylldrige lksgdacvae yqqagrgrg
 121 rkwsfpfgan lylsmfwrle qgpaaigls lvgivmaev lrklgadvr vkwpndlylq
 181 drklagilve ltgktgdaaq ivigaginma mrrveesvvn qgwitlqeag inldrntlaa
 25 241 mlirelraal elfeqeglap ylsrwekldn finrpvklii gdkeifgisr gidkqgalll
 301 eqdgiikpwm ggeislrsae k

30 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing

from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for
5 carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A producer cell for the production of a viral vector, which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, thus producing a biotin/desthiobiotin-tagged viral vector.

2. A cell for the production of a protein of interest (POI), which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, thus producing a biotin/desthiobiotin-tagged POI.

3. A cell for the production of an untagged entity of interest (EOI), which cell is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, such that an adventitious agents produced by the cell will be biotin/desthiobiotin-tagged and may therefore be distinguished from the EOI.

4. A cell which is capable of converting 7,8-Diaminoperlargonate (7-DAPA) or 7-keto-8-aminopelargonate (7-KAPA) into biotin or desthiobiotin, such that in the presence of 7-DAPA or 7-KAPA, the cell produces biotin/desthiobiotin-tagged cell surface proteins.

5. A cell according to any preceding claim, which expresses an enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin.

6. A cell according to claim 5, wherein the enzyme is the E. Coli BioD protein or a homolog thereof.

7. A cell according to any preceding claim, which expresses two enzymes:

a first enzyme capable of catalysing the conversion of 7-keto-8-aminopelargonate (7-KAPA) into 7,8-Diaminoperlargonate (7-DAPA); and

a second enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin.

5

8. A cell according to claim 7, wherein the first enzyme is BIO1 or a homolog thereof and the second enzyme is BIO3 or a homolog thereof, and the cell expresses a BIO3-BIO1 fusion protein.

10 9. A cell according to any preceding claims, which expresses three enzymes:

a first enzyme capable of catalysing the conversion of 7-keto-8-aminopelargonate (7-KAPA) into 7,8-Diaminoperlargonate (7-DAPA);

a second enzyme capable of catalysing the conversion of 7,8-Diaminoperlargonate (7-DAPA) into desthiobiotin; and

15 a third enzyme capable of catalysing the conversion of desthiobiotin into biotin.

10. A cell according to claim 9, wherein the first enzyme is BioA or a homolog thereof and the second enzyme is BioD or a homolog thereof, the third enzyme is
20 BioB or a homolog thereof and the cell expresses a BioA-BioD-BioB fusion protein.

11. A cell according to any preceding claim which expresses a polyhistidine tag, resulting in the production of a polyhistidine/desthiobiotin dual tagged viral
25 vector/POI.

12. A cell according to any preceding claim, which expresses a ligase enzyme which couples biotin/desthiobiotin to a biotin/desthiobiotin acceptor sequence.

30 13. A cell according to claim 12, wherein the ligase enzyme is BirA or a homolog thereof.

14. A method for producing a cell according any preceding claim, which comprises the step of introducing a nucleotide sequence which encodes an enzyme which catalyses the conversion of 7,8-Diaminoperlargonate (7-DAPA) or (7-KAPA) into desthiobiotin into the cell.

5

15. A method for producing a biotin- or desthiobiotin-tagged viral vector which comprises the step of production of the viral vector using a producer cell according to claim 1, in the presence of 7-DAPA or 7-KAPA.

10

16. A method for producing a biotin- or desthiobiobiotin-tagged protein of interest (POI) which comprises the step of expression of the POI from a cell according to claim 2, in the presence of 7-DAPA or 7-KAPA.

15

17. A method for purification of a viral vector/protein of interest (POI), which comprises the following steps:

(i) producing a viral vector by a method according to claim 15, or a POI by a method of claim 16;

(ii) avidin-based capture of the vector/POI; and

(iii) elution of the captured vector/POI.

20

18. A kit for the production of a cell according to any of claims 1 to 13, which comprises:

(i) a plasmid comprising a nucleotide sequence encoding an enzyme capable of synthesising desthiobiotin from 7-DAPA, such as bioD, BIO3 or a homolog or ortholog thereof;

25

and any one or more of the following:

(ii) a plasmid comprising a nucleotide sequence encoding an enzyme capable of converting 7-KAPA to 7-DAPA, such as bioA, BIO1 or a homolog or ortholog thereof;

30

(iii) a plasmid comprising a nucleotide sequence encoding an enzyme capable of converting desthiobiotin to biotin, such as bioB, BIO2 or a homolog or ortholog thereof; and

(iv) a plasmid comprising a nucleotide sequence encoding a ligase enzyme capable of covalently coupling desthiobiotin to an acceptor site, such as BirA or a homolog or ortholog thereof.

5 19. A kit according to claim 18 for the production of a producer cell according to claim 1 which also comprises any one or more of the following:

(v) a plasmid comprising a viral genome;

(vi) one or more plasmids encoding proteins required by the producer cell in order to produce a viral particle, such as Gag, Pol and/or Env;

10 (vii) a plasmid encoding a desthiobiotin acceptor sequence, optionally as part of or fused to an exogenous cell membrane protein; and

(viii) a plasmid encoding a His₆ sequence, optionally as part of or fused to an exogenous cell membrane protein.

15 20. A kit according to claim 18 for the production of a cell according to claim 2, which also comprises:

(v) a plasmid comprising a nucleotide sequence encoding the POI, optionally joined to a nucleotide sequence encoding a desthiobiotin acceptor and/or a nucleotide sequence encoding a His₆ sequence.

20

21. A producer cell for producing a viral vector which comprises a desthiobiotin-tagged cell surface protein, which co-packages with the viral vector causing the viral vector produced by the cell to be desthiobiotin-tagged.

25 22. A method for purification of an entity of interest (EOI) which comprises the following steps:

(i) production of the EOI using a cell according to claim 3, in the presence of 7-DAPA or 7-KAPA; and

(ii) avidin-based removal of the tagged adventitious agent.

30

23. A cell according to claim 3 or a method according to claim 22, wherein the EOI is an antibody and the cell is a hybridoma cell line.

24. A cell according to claim 3 or a method according to claim 22, wherein the adventitious agent is an enveloped virus.

5 25. A method for producing a biotin- or desthiobiotin-coated cell, which comprises the step of culturing a cell according to claim 4 in the presence of 7-DAPA or 7-KAPA.

26. A biotin- or desthiobiotin-coated cell according to claim 4.

10

27. A cell according to claim 26 which is labelled with magnetic avidin/streptavidin beads or a fluorescent label or is attached to a solid support.

15 28. The use of a cell according to claim 27 in cell patterning, tissue culturing or cell-screening.

Figure 1

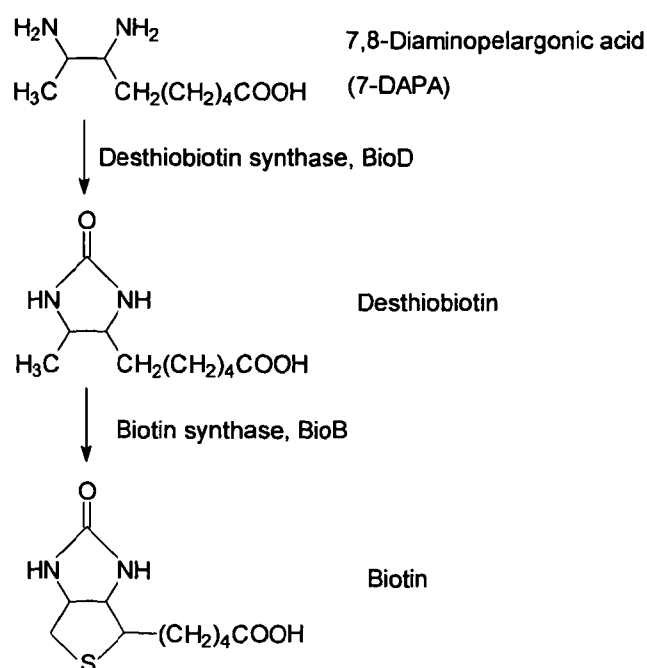


Figure 2

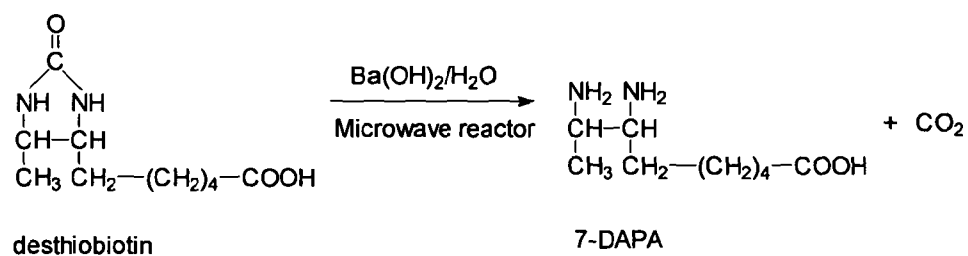


Figure 3A

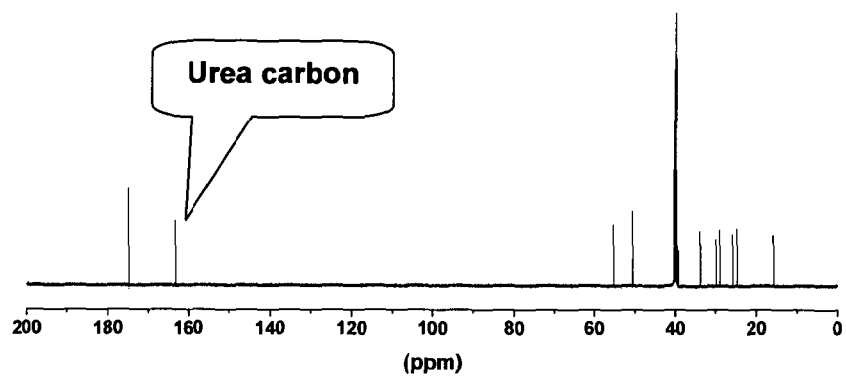


Figure 3B

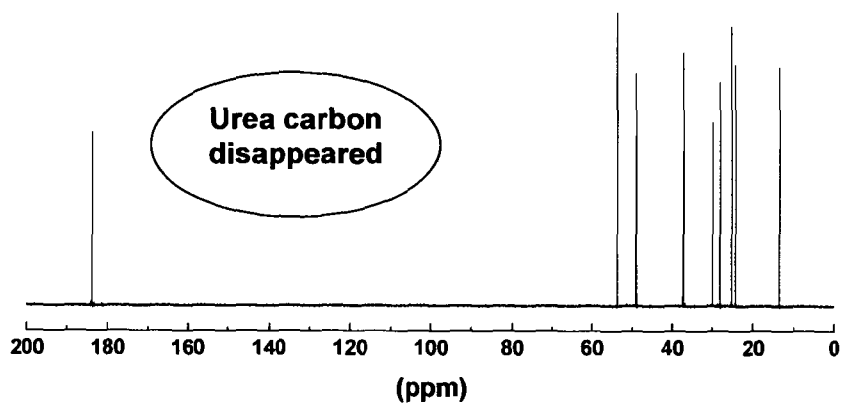


Figure 4

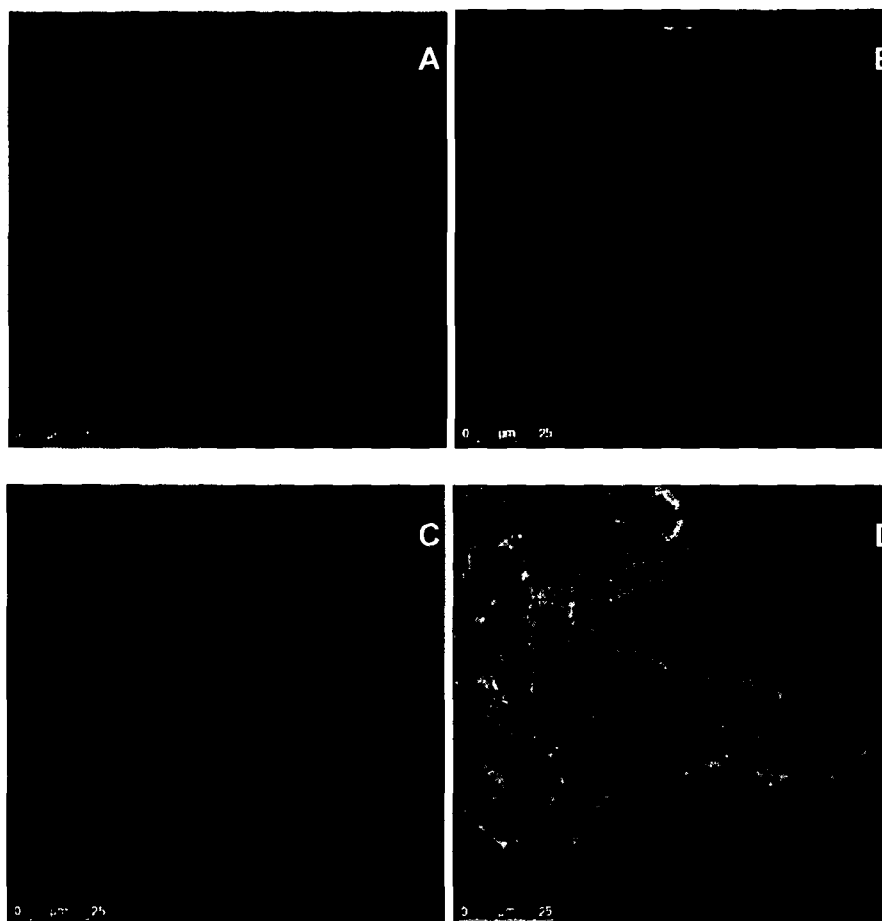


Figure 5A

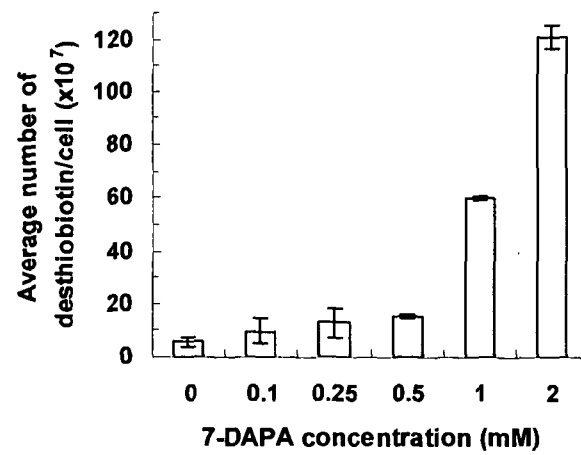


Figure 5B

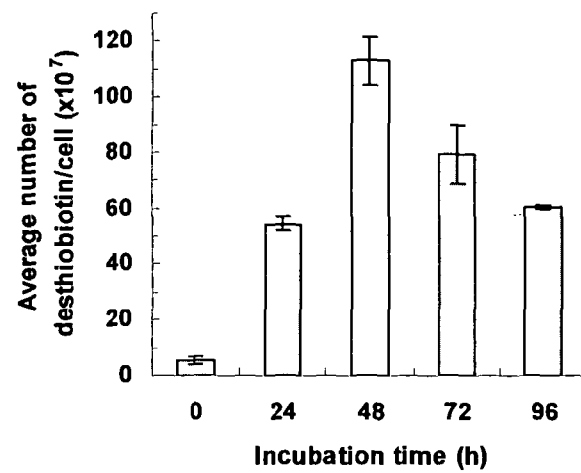


Figure 6A

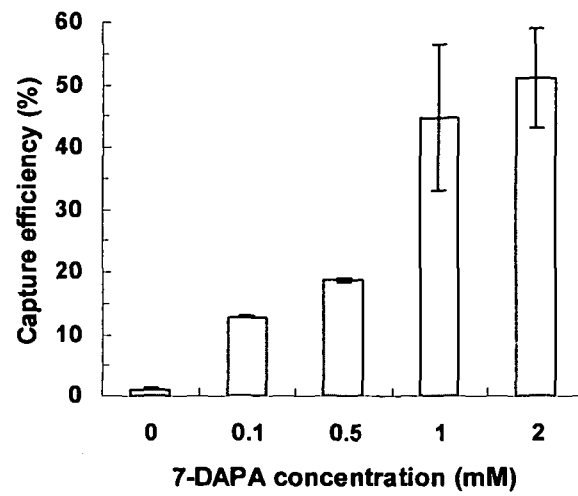


Figure 6B

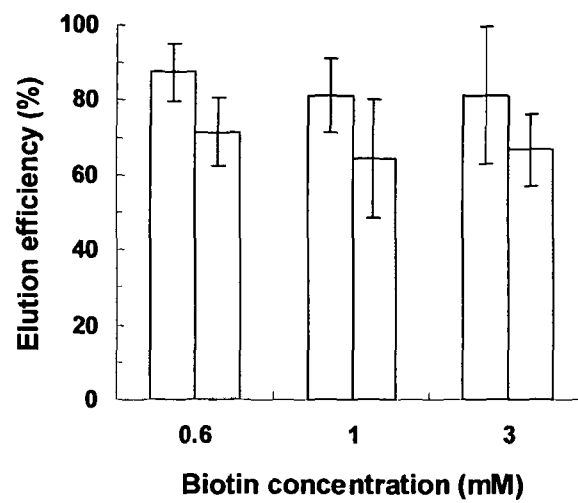


Figure 7A

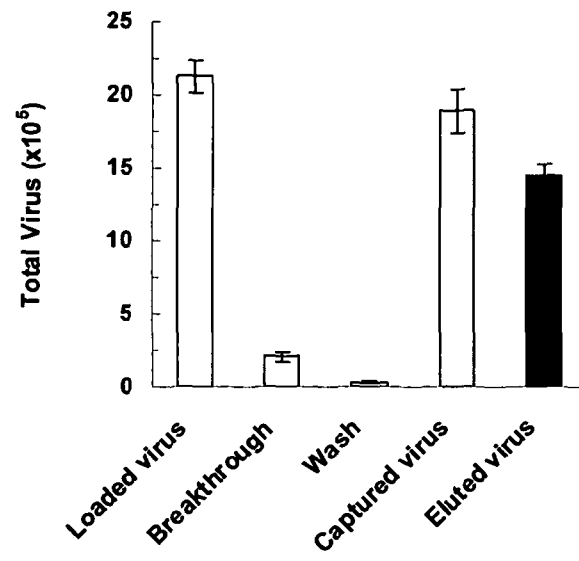
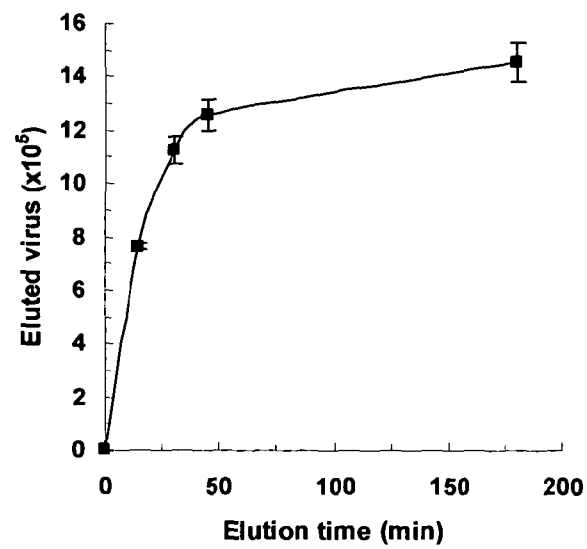


Figure 7B



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/002182

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N15/867 C12N15/86 C12N15/62 C12N9/00 C12N7/02 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
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☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 11 March 2011		Date of mailing of the international search report 21/03/2011
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Chambonnet, F

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