

SINGLE RECOMBINATION SYSTEM AND METHODS OF USE

The present invention relates to a single recombination system for the cloning of recombinant poxviruses by a single recombination event in a modified poxvirus vector. It further relates to the modified poxvirus vectors, uses thereof and a method for the production of recombinant poxviruses.

BACKGROUND OF THE INVENTION

Recently, significant effort has been invested in developing and improving recombinant poxvirus-based technologies. Poxvirus-based vectors have been identified as beneficial for a number of uses such as generating immune responses through vaccines, in the development of new vaccine therapies, and use in gene therapy applications. The advantages associated with recombinant poxvirus vectors are well-known and include efficient delivery of genetic material to multiple cell types; generous levels of protein expression; and the ability to elicit cell-mediated immune responses in addition to antibody-based responses.

Poxviruses are well-known cytoplasmic viruses, thus any genetic material carried by such viral vectors normally remains in the cytoplasm without the disadvantage of inadvertent integration into the host cell genome. Poxviruses can be readily genetically engineered to contain and express foreign genes that have been inserted into their genome using, for example, cloning techniques such as homologous recombination. These foreign genes can encode a wide range of proteins, such as antigens that induce protection against one or more infectious agents, immune modulating proteins such as co-stimulatory molecules, or enzymatic proteins. For example, recombinant poxviruses have been engineered to express immunizing antigens of herpes virus, influenza, and human immunodeficiency virus (HIV).

One of the main advantages of poxviruses as vectors is their large genome size, which permits the insertion of a large amount of heterologous genetic material including, for example, multiple genes (i.e., as a multivalent vector). However, the heterologous genetic material must be inserted at an appropriate site within the pox genome for the recombinant virus to remain viable. Thus, the genetic material must be inserted at a site in the viral DNA which is non-essential.

A well-established approach for the cloning of recombinant poxviruses is based on two separate recombination events. During the first recombination step, a gene of interest and a reporter and/or marker cassette are integrated into a viral genome. For the selection process, an

antibiotic resistance gene is commonly used. Following the subsequent isolation of recombinant poxviruses from the pool of recombinant and non-recombinant poxviruses using the selection/marker cassette, the selection and marker cassette should be deleted if the recombinant poxvirus is intended for use as, for example, a vaccine in humans. For this purpose, a second
5 recombination event must be performed involving further passaging and plaque purification of the recombinant poxvirus. Consequently, presently known techniques for cloning recombinant poxviruses are usually time-consuming and laborious endeavors, especially when compared to those procedures commonly used for the cloning of other types of recombinant expression vectors.

10 Accordingly, there is a need in the art for improved cloning systems and methods for the efficient generation of recombinant poxviruses.

SUMMARY

The present disclosure is based on a modified poxvirus vector that allows for the generation of recombinant poxviruses by a single recombination event. The modified viral vector of the disclosure comprises a reporter gene that is deleted if a recombination with the insert of interest successfully occurs.

In a first aspect, there is provided a modified poxvirus vector comprising a reporter gene and a selection component comprising a selection gene whose expression is regulated by the tetracycline operator/repressor system located between a pair of flanking sequences allowing for homologous recombination.

In one embodiment, the present disclosure is directed to a modified poxvirus vector comprising at least one reporter gene located between two flanking sequences for homologous recombination. In one embodiment, the modified poxvirus vector further comprises at least one selection component located between the two flanking regions for homologous recombination in a permissive host cell. In another embodiment, the selection component comprises a selection gene that inhibits or slows down poxvirus replication in the host cell or is cytotoxic to the host cell. In another embodiment, the reporter gene and the selection component are located between a single pair of flanking sequences allowing for homologous recombination. In yet another embodiment, the reporter gene and the selection component are located between more than one pair of flanking sequences allowing for homologous recombination. In a further embodiment, the selection component comprises a selection gene whose expression is inducible. In a specific embodiment, the reporter gene codes for a fluorescent protein, for example, green fluorescent protein. In a further specific embodiment, the selection component comprises a selection gene selected from a gene coding for a DNase, a RNase, or a protease. In a further specific embodiment, expression of the selection gene is under the control of a regulatory sequence, preferably a promoter. In another specific embodiment the selection gene is regulated by an inducible expression system, for example, the tetracycline operator/repressor (TetO2/TetR) system.

In another embodiment, the present disclosure is directed to a recombinant poxvirus generated using a modified poxvirus vector of the present disclosure.

In another embodiment, the present disclosure is directed to a host cell comprising a modified poxvirus vector of the present disclosure. In a further embodiment, the present disclosure is directed to a host cell comprising a recombinant poxvirus generated using a modified poxvirus vector of the disclosure.

In a second aspect, there is provided a cell comprising the modified poxvirus vector as defined above.

In a third aspect, there is provided the use of the modified poxvirus vector as defined above or the cell as defined above to generate a recombinant poxvirus.

In a further embodiment, the disclosure provides a method of using the modified poxvirus vector to generate a recombinant poxvirus. In another embodiment, the present disclosure is directed to a method for the generation of a recombinant poxvirus that comprises infecting a permissive

host cell with a modified poxvirus vector according to the present disclosure, and subsequent transfection of the host cell with a plasmid comprising heterologous genetic material of interest, under conditions that permit homologous recombination between the vector and plasmid. In one embodiment, the method comprises inducing expression of the selection gene. In a further embodiment, the method comprises separating host cells comprising the recombinant poxvirus from host cells comprising non-recombinant poxvirus. In a further embodiment, the method comprises using permissive host cells comprising the recombinant poxvirus for at least one further passage in previously uninfected permissive host cells.

In a fourth aspect, there is provided a method for generating a recombinant poxvirus capable of expressing a gene of interest comprising the steps of:

- 1) infecting a permissive host cell with the modified poxvirus vector as defined above; and
- (2) transfecting said infected permissive host cell with a plasmid comprising said gene of interest under conditions that permit homologous recombination between said vector and said plasmid,

whereby a recombinant poxvirus is produced.

In a further embodiment, the present disclosure is directed to the use of the modified poxvirus vector according to the disclosure for the generation of a recombinant poxvirus.

In a fifth aspect, there is provided a cell comprising a recombinant poxvirus generated by the method as defined above.

In a sixth aspect, there is provided the use of the method as defined above or the cell as defined above to generate a recombinant poxvirus.

In a seventh aspect, there is provided a recombinant poxvirus generated using the method as defined above or the cell as defined above.

In an eighth aspect, there is provided the modified poxvirus as defined above, the cell as defined above or the recombinant poxvirus as defined above as a medicament.

In a ninth aspect, there is provided the modified poxvirus as defined above, the cell as defined above or the recombinant poxvirus as defined above as a therapeutic or prophylactic vaccine for the treatment or prevention of cancer, influenza, hepatitis, AIDS, mumps, rabies, encephalitis, stomach or duodenal ulcers, malaria, sleeping sickness, lyme disease, reactive arthritis, pneumonia, leprosy, diphtheria, candidiasis and/or toxoplasmosis.

In a tenth aspect, there is provided a nucleic acid or its complement having at least 70% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 20-23.

In particular embodiments, the modified poxvirus vector according to the disclosure is a vaccinia virus. In a further particular embodiment, the modified poxvirus vector according to the disclosure is a Modified Vaccinia Ankara virus (MVA). In a further embodiment, the present disclosure is directed to the modified poxvirus according to the disclosure, the recombinant poxvirus according to the disclosure, and a cell according to the disclosure comprising the modified or recombinant poxvirus as a medicament. In specific embodiments the present disclosure is directed to the modified poxvirus according to the disclosure, the recombinant poxvirus according to the disclosure, and a cell according to the disclosure comprising the modified or recombinant poxvirus as a therapeutic or prophylactic vaccine for the treatment or prevention of cancer, influenza, hepatitis, AIDS, mumps, rabies, encephalitis, stomach or duodenal ulcers, malaria, sleeping sickness, lyme disease, reactive arthritis, pneumonia, leprosy, diphtheria, candidiasis and/or toxoplasmosis. In a specific embodiment, the medicament is a therapeutic or prophylactic vaccine. In another specific embodiment, the medicament is a therapeutic or prophylactic vaccine for the treatment of cancer.

Other embodiments of the disclosure are provided by the detailed description, the Examples, and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the reporter/selection cassette of an exemplary modified poxvirus vector according to the invention. The vector is based on the MVA genome (Gene Bank Accession # U94848; Antoine, G., Scheiflinger, F., Dorner, F. and Falkner, F.G. "The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses", Virology 244 (2), 365-396 (1998)) into which the reporter/selection cassette was introduced into the insertion site (Deletion III). Flank1 Del3 = flanking sequences upstream of the Deletion III site of the MVA genome; Ps promoter = strong synthetic promoter; TetO2 = Tetracycline Operator (the binding site for TetR); TetR = Tetracycline Repressor; Flank2 Del3 = flanking sequences downstream of the deletion III site of the MVA genome.

FIG. 2 is a schematic representation of the process of homologous recombination in host cells permissive for the poxvirus. For a MVA poxvirus such host cells include, for example, chicken embryo fibroblasts (CEF). Following infection of cells with the modified poxvirus vector of the present invention, the cells are transfected with a plasmid that contains the genetic material of interest (e.g. LacZ) located between two flanking sequences for homologous recombination (Flank 1/2 Del3). A recombinant poxvirus is produced where the homologous flanks recombine resulting in incorporation of the genetic material of interest, and deletion of the reporter/selection cassette.

FIG. 3 provides a map of the exemplary plasmid (vEM07; SEQ ID NO: 1) used for inserting genetic material of interest into the MVA genome, as schematically shown in FIG. 2. LacZ = Bacterial gene coding for β -Galactosidase.

FIG. 4 schematically depicts the resulting fusion of the tetracycline operator (TetO2) to the DNase gene achieved by PCR.

FIG. 5 schematically depicts the resulting fusion of the Ps promoter to the TetO2-DNase fragment.

FIG. 6 shows a map of the standard recombination plasmid vEM11 used for cloning recombinant MVA.

FIG. 7 shows a map of the recombination plasmid vEM12. The Ps-TetO2-DNase cassette was cloned in the vEM11 plasmid via the SacI and SpeI restriction sites. BsdR = Blasticidine resistance gene.

FIG. 8 schematically depicts the fusion of the Ps promoter to the tetracycline repressor coding region (TetR).

FIG. 9 shows a map of the recombination plasmid vEM31. The Ps-TetR cassette was cloned in the vEM12 plasmid via the SacI and NheI enzymatic restriction sites.

5 FIG. 10 illustrates the effects of inducible DNase expression on MVA replication. CEF cells were infected with the recombinant MVA mEM07 (7.5 = 7.5 promoter), mEM06 (Ps = Ps promoter), mEM08 (H5 = H5 promoter) and incubated with media containing different concentrations of tetracycline (Tet 0 = no tetracycline; Tet 25 = 25 μ tetracycline per ml). As a control, CEF cells were infected with MVA empty vector (MVA). One set of samples were
10 subsequently transfected with vEM12 (MVA + DNase). After 48 hours of incubation at 37°C, 5% CO₂ cells were harvested and the virus titer was determined. CEF cells infected with MVA empty vector were also incubated with tetracycline (MVA + Tet). TCID₅₀= Tissue Culture Infectious Dose 50.

FIG. 11 depicts the resulting PCR analysis of the Deletion III site. The insertion site for
15 foreign genes in the MVA genome (deletion 3) was investigated using PCR.

DETAILED DESCRIPTION

The present invention is based on a modified vector derived from a poxvirus that permits the generation of recombinant poxviruses by means of a single recombination event. The modified viral vector of the invention comprises a reporter gene that is deleted if a recombination with the insert of interest successfully takes place. Thus, the modified viral vector of the present invention provides for the detection of a single recombination event which simultaneously deletes the reporter/selection cassette and inserts the heterologous genetic material of interest in the recombinant viral vector.

Definitions

The term "vector" as used herein means any genome or part or fragment thereof of a poxvirus that contains sufficient genetic information to allow reproduction of the poxvirus in a permissive host cell. The vector according to the present invention can be a genetically engineered vector, such that it may only contain part or all of the elements of the poxvirus it is derived from, and/or additional genetic elements. In a specific embodiment, the vector according to the present invention is the genome or part or fragment thereof of the Modified Vaccinia Ankara (MVA) virus. In further specific embodiments, the vector according to the present invention is the genome or part thereof of an MVA virus selected from the group consisting of MVA-F6 (e.g. Lotz *et al.* "Partial tyrosinase-specific self tolerance by HLA-A*0201-restricted cytotoxic T lymphocytes in mice and man", Intern. J. of Cancer 2003; 108 (4): 571-79), MVA575 (ECACC deposit number V00120707), MVA-M4 (Ober *et al.* "Immunogenicity and safety of defective Vaccinia virus lister: Comparison with modified Vaccinia virus Ankara", J. Virol 2002; 76 (15): 7713-23), Acam3000 (Accession number AY603355), MVA-ATCC (ATCC VR-1508), MVATGN33.1 (Accession number EF675191), MVA-I721 (Accession number DQ983236).

In another embodiment of the invention, the vector according to the present invention is the genome or part thereof of a poxvirus selected from the group consisting of Vaccinia virus Western Reserve (Accession number NC006998), Vaccinia virus Wyeth (e.g. Fogg *et al.* "Virus induced by Vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions", J. Virol. 2004; 78 (19): 10230-37), Vaccinia virus Lister (Accession number DQ191324), NYCBH (New York City Board of Health); CDC (Centers for Disease Control and Prevention), Vaccinia (smallpox) vaccine recommendations of the Immunization Practices Advisory Committee (ACIP), *Morbidity and Mortality Weekly Report* 40(R14): 1-10, 1991.), Canary

pox (Accession number 005309 and AY318871), Fowlpox (Accession number 581527), Vaccinia virus Copenhagen (Accession number M35027) NYVAC, ALVAC, TROVAC (Paoletti *et al.* "Highly attenuated poxvirus vectors: NYVAC, ALVAC, TROVAC", Dev. Biol. Stand. 1995, 84: 159-63).

5 The term "reporter gene" as used herein means a gene conferring certain characteristics on cells or organisms expressing them which can be readily detected, identified or measured. Reporter genes are commonly used to determine whether a gene of interest has been incorporated or is expressed by a cell or organism population. In the context of the present invention, detection of the expression product from the reporter gene indicates that no
10 homologous recombination has occurred, and that the gene insert of interest has not been successfully inserted into the viral vector. Generally, any reporter gene can be used that permits a separation of cells comprising the original vector of the invention and those cells comprising a vector where homologous recombination has occurred. Non-limiting examples of reporter genes that are suitable for use in the present invention include reporter genes that can induce
15 visually identifiable characteristics (e.g. involving fluorescence), which enables separation by, for example, FACS and luminescent proteins such as the gene coding for jellyfish green fluorescent protein (GFP), the gene coding for the enhanced GFP (eGFP), mPlum, mCherry, tdTomato, mStrawberry, mRaspberry, mRFP1, mTangerine, mYFP (Tsien), J-Red, AceGFP, CopGFP, HcRed-tandem, PhiYFP (Evrogen), DsRed, DsRed2, DsRed-Express, DsRed-monomer,
20 EGFPAcGFP1, AmCyan1, AsRed2, EBFP, HcRed1, ZsYellow1 (Clontech), mKO, Azami-Green, mAG, Kaede, MiCy (MBL Intl.), Venus (Miyawaki), Ypet, CyPer (Dougherty), EYFP, Emerald (Invitrogen), Cerulean (Priston), T-Sapphire (Griesbeck), AQ143 (Lukyanov), cOFP (Stratagene), eqFP611 (Weidenmann), Renilla GFPs (various providers, e.g. Stratagene), the luciferase gene or the lacZ gene. In one embodiment of the invention, the reporter gene is a
25 fluorescence coding gene.

 Further non-limiting examples of reporter genes include genes coding for proteins for which specific antibodies exist and that are displayed on the surface on the host cell. Such reporter genes permit the separation of cells comprising the original vector of the invention and cells comprising a vector in which homologous recombination has taken place by affinity
30 purification, for example, by columns comprising antibody covered resin or magnetic beads covered with said antibodies, wherein the antibodies are specific for the protein encoded by the reporter gene. In a specific embodiment, the reporter gene codes for a fusion gene of a cell surface receptor and an affinity tag so that the affinity tag is displayed on the cell surface. The

skilled person is aware of a variety of affinity tags and corresponding ligands that are commonly used. In further specific embodiments, the gene codes for a fusion gene of a cell surface receptor and the fused affinity tag is selected from the group consisting of a FLAG-tag (N-DYKDDDDK-C), an epitope tag, the V5-tag, the c-myc-tag, the His-tag and/or the HA-tag. In
5 a further embodiment, the reporter gene codes for a small viral surface protein selected from the group consisting of Alphavirus E1 and E2, Flavivirus E1 and E2, Coronavirus S, HE, M and E, Arterivirus GP, Rhabdovirus G, Filovirus GP, Paramyxovirus F, HN, H and G, Orthomyxovirus M2, NA, HA and HEF, Bunyavirus Gn and Gc, Arenavirus GP, Bornavirus G, Retrovirus Env, Hepadnavirus S, M and L.

10 The term "selection component" as used herein refers to any nucleic acid that may be utilized to exert a selection pressure on a host cell comprising such a selection component. In one embodiment, selection pressure is exerted by expression of a selection gene that inhibits or retards virus replication in the host cell. In another embodiment, selection pressure is exerted by expression of a selection gene that leads to a dying off of the host cell. In one embodiment, the
15 selection component comprises at least one selection gene whose expression is inducible. In a specific embodiment, the induction of the expression is controlled by means of regulatory sequences, for example, a promoter. In a further embodiment, the selection component further comprises a bacterial inducible system that regulates expression of the selection gene. In one specific embodiment, the bacterial inducible system features the selection gene under the control
20 of a promoter that is fused to a repressor binding site, wherein the selection component further comprises a gene coding for a repressor that binds to the repressor binding site. In a further specific embodiment, the repressor binding site is the tetracycline repressor binding site/operator (TetO2) and the gene coding for a repressor is the tetracycline repressor gene (TetR), which can be isolated from the pcDNA6TR plasmid (available from Invitrogen). In
25 further embodiments, the selection component further comprises a mammalian inducible system that regulates the expression of the selection gene. In a specific embodiment, the mammalian inducible system is the RheoSwitch (New England Biolabs) system. In further specific embodiments, the inducible system is selected from the group consisting of the LentiX system (Clontech), the Q-mate system (Krackeler Scientific, Inc.), the Cumate-inducible expression
30 system (NRC Canada), and/or the Genostat system (Upstate).

In a further embodiment of the invention, the selection gene is a cytotoxic gene. The term "cytotoxic gene" as used herein refers to any gene that, upon expression, will lead to the loss or complete apoptosis of the host cell, wherein said loss or apoptosis of the host cell is

caused by the expressed gene product itself and only involves the direct interactions between the expressed gene product and naturally occurring components of the host cell (e.g. the host cell's DNA, RNA, proteosome, membranes or metabolites). In specific embodiments, the cytotoxic gene codes for a DNase (e.g. available from Invitrogen; ORF clone pENTR221, clone ID
 5 IOH23149), an RNase, a protease, an ion channel, or an apoptosis inducer, such as caspase and/or ROS.

The term "insert" or "insert of interest" as used herein refers to any nucleic acid that is to be introduced into the vector of the invention by homologous recombination. In one embodiment, the insert comprises at least one heterologous gene flanked by flanking sequences
 10 that allow homologous recombination to take place with the vector of the invention. In a specific embodiment of the invention, at least one heterologous gene is under the control of a regulatory sequence, preferably a promoter.

An example of an insert of interest is depicted in FIG. 2. Here, the insert is comprised of the plasmid vector that is used to transfect the host cell and comprises the two flanking regions
 15 and the LacZ gene under control of the lac promoter. In a specific embodiment, the heterologous gene codes for at least one antigen, preferably an antigen that is capable of inducing an immune response in a patient. In specific embodiments, the heterologous gene codes for an antigen selected from the group consisting of an antigen of a heterologous virus, an antigen of a bacterium, an antigen of a prokaryote, an antigen of a fungus, and/or an antigen of a
 20 helminth. In particular embodiments, the heterologous gene codes for an antigen of a species selected from the group consisting of Influenza virus A, B, C, Hepatitis virus A, B, C, E, Human Immunodeficiency virus, Rubella virus, Mumps virus, Rabies virus, Human papilloma virus, Epstein Barr virus, Tickborne virus, Crimean Kongo Fever virus, Ebola virus, Nipah virus, Dengue virus, Chikungunya virus, Enterovirus, West Nile virus, Rift Valley Fever virus, Japanese
 25 encephalitis virus, Hantavirus, Rotavirus, SARS Coronavirus, New emerging viruses, Chlamydia trachomatis, Clostridium botulinum, Clostridium tetani, Bacillus anthracis, Legionella pneumophila, Neisseria meningitidis (Menigococcus), Yersinia pestis, Mycobacterium tuberculosis, Mycobacterium leprae, Salmonella typhi, Listeria monocytogenes, Vibrio cholerae, Haemophilus influenzae, Bordetella pertussis, Helicobacter pylori, Borrelia spp. (recurrentis,
 30 hispanica, parkeri, burgdorferi), Leptospira interrogans, Rickettsia spp., Coxiella burnettii, Mycoplasma pneumonia, Corynebacterium diphtheriae, Treponema pallidum, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Entamoeba histolytica, Giardia intestinalis, Trypanosoma brucei, Leishmania spp., Histoplasma capsulatum, Aspergillus

spp., *Candida albicans*, *Cryptococcus neoformans*, *Pneumocystis carinii*, *Wuchereria bancrofti*, *Schistosoma mansoni* and/or *Toxoplasma gondii*.

In a further specific embodiment, the heterologous gene codes for at least one antigen that induces a protective or therapeutic immune response against infectious diseases (e.g. viral and bacterial surface proteins) or against cancer cells, for example, cells of cervical carcinoma, melanoma, multiple myeloma, breast cancer, prostate cancer, follicular B-cell non-Hodgkins lymphoma and/or kidney cancer.

Vectors of the present invention

In a first aspect, the present invention relates to a modified poxvirus vector comprising at least one reporter gene located between two flanking sequences for homologous recombination. The reporter gene may be replaced by an insert of interest through homologous recombination in a host cell infected with the modified poxvirus vector. Therefore, host cells comprising a modified poxvirus vector of the present invention, in which homologous recombination has taken place, can be distinguished from those host cells comprising a modified poxvirus vector where such a homologous recombination has not taken place. This can be accomplished by means of detecting the protein coded for by the reporter gene. For example, if the reporter gene encodes the GFP or the azami green protein, then host cells comprising a modified poxvirus vector of the present invention in which homologous recombination has taken place will not produce the GFP or azami green protein, because the genetic information for the GFP or azami green protein has been removed from the vector by the homologous recombination event and has been replaced with the insert of interest. Accordingly, such host cells will not display fluorescence upon excitation and can, for example, be separated from those cells that do fluoresce by fluorescence activated cell sorting (FACS).

In one embodiment, the reporter gene is under the control of a promoter, preferably a virus promoter, more preferably a vaccinia virus promoter or a synthetic promoter. In another embodiment, the promoter is a strong promoter, preferably a strong synthetic promoter. In a specific embodiment, the promoter is the P_s promoter having the sequence AAAAATTGAAATTTTATTTTTTTTTTTTGGGAATATAAATA (Sekhar Chakrabarti, Jerry R. Sisler and Bernard Moss: Compact, synthetic, Vaccinia Virus Early/Late Promoter for Protein Expression. *Biotechniques* 23:1094-1097, 1997). In another specific embodiment the promoter is the modified H5 promoter having the sequence AAAAAATGAAAATAAATACAAAGGTT

CTTGAGGGTTGTGTAAATTGAAAGCGAGAAATAATCATAAAATT; Rosel JL, Earl PL, Weir JP, Moss B, "Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the HindIII H genome fragment", J. Virol. 60 (2): 236-249, 1986).

5 In a further embodiment, the reporter gene encodes a fluorescent protein. "Empty" vectors (i.e., vectors in which no recombination events have taken place) express the reporter gene, and therefore cells comprising such vectors can fluoresce. Homologous recombination in a permissive cell leads to deletion of the reporter gene, and the procedure results in recombinant
10 vectors containing the insert of interest that do not cause fluorescence in the host cell. In specific embodiments, the fluorescent protein is GFP, enhanced GFP (eGFP), or the azami green protein (available from MBL International Inc., Woburn, Massachusetts, USA and distributed by MoBiTec GmbH, Goettingen, Germany).

In a further aspect, the vector of the present invention further comprises at least one selection component that is similarly located between the two flanking sequences for
15 homologous recombination. In one embodiment, the reporter gene and the selection component are located between more than one pairs of flanking sequences allowing for homologous recombination. In another embodiment, the selection component comprises a selection gene whose expression is inducible. In a further embodiment, the selection component further comprises a bacterial inducible system that regulates expression of the selection gene. In one
20 specific embodiment, the bacterial inducible system features the selection gene under the control of a promoter that is fused to a repressor binding site, wherein the selection component further comprises a gene coding for a repressor that binds to the repressor binding site. In a further specific embodiment, the repressor binding site is the tetracycline repressor binding site/operator (TetO2) and the gene coding for a repressor is the tetracycline repressor gene
25 (TetR), which can be isolated from the pcDNA6TR plasmid (available from Invitrogen). Upon transfection of the host cell, the repressor is expressed in the cell and binds to the repressor binding site of the selection component upstream to the selection gene to thereby suppress expression of the selection gene. Once tetracycline is added to the system, it binds to the tetracycline repressor and immediately detaches from the binding site on the DNA which, in
30 turn, leads to the expression of the selection gene. Consequently, expression of the selection gene is dependent on the addition of tetracycline and, therefore, expression of the selection gene is rendered inducible. In a further embodiment, the promoter that controls expression of the selection gene and the gene coding for the repressor, is a virus promoter, a vaccinia virus

promoter or, most preferably, a strong synthetic promoter, such as the Ps promoter or the modified H5 promoter.

In a further embodiment of the invention, the selection component comprises a selection gene that, upon expression, inhibits or retards virus replication in the host cell. If the selection gene is induced, this allows for an improved selection of viruses comprising the vector of the invention where homologous recombination has taken place, and the selection component and the reporter gene have been replaced with the insert of interest. Consequently, host cells comprising a vector in which homologous recombination has taken place, harbor more replicated recombinant viruses than those where no homologous recombination has taken place. Furthermore, the inhibitor of viral replication is expressed, leading to a decreased or even complete prohibition of viral replication. Thus, the ratio of produced viruses comprising a genome including the insert of interest to those viruses comprising the genome without said insert of interest is significantly shifted in favor of the former. Accordingly, as a percentage, the system can be manipulated to generate an improved yield of viruses comprising the insert of interest. In particular embodiments, the selection gene is an RNase, an ion channel or, a DNase (available from Invitrogen; ORF clone pENTR221, clone ID IOH23149).

In a further embodiment of the invention, the selection component is a cytotoxic gene, i.e. any gene that, upon expression, will lead to the loss or complete apoptosis of the host cell. In specific embodiments, the cytotoxic gene codes for a DNase, an RNase, a protease, an ion channel, or an apoptosis inducer.

According to the invention, the selection component is an optional embodiment of the invention, since host cells harboring a recombinant vector of the invention comprising the insert of interest can be selected and isolated by means of the reporter gene alone. While the reporter gene is essential for the detection and/or selection of such a recombinant vector, the optional inducible expression of the selection component can be used for, for example, single or multiple passages of vector production.

In particular embodiments, the selection component may, for example, significantly accelerate the generation of recombinant poxviruses comprising the insert of interest, as host cells not harboring a recombinant vector of the invention comprising said insert are directed to produce less or no viruses (or are killed entirely) upon induction of the selection component. In this case, the recombinant vectors of the invention, containing a reporter gene and a selection component result in fluorescence expressing "empty" vectors sensitive against tetracycline and

the tetracycline-insensitive recombinant vectors devoid of any fluorescence. In other words, only cells containing empty vector exhibit fluorescence and thus the non-recombinants can be separated by techniques including plaque picking or FACS sorting. If the expression of the selection component is induced beforehand, the separation can be carried out in a significantly improved manner, since the number of cells harboring "empty" vectors is dramatically and beneficially reduced prior to separation.

In further embodiments of the present invention, flanking regions permit homologous recombination. Homologous recombination generally involves the alignment of similar sequences, formation of a Holliday junction, and breaking and repair (known as resolution) of the nucleic acid resulting in an exchange of material between the strands of the nucleic acids. Therefore, any flanking regions that are similar enough to allow for homologous recombination are encompassed by the present invention. In a specific embodiment, the flanking regions are identical. In a further specific embodiment, the flanking regions have a size of 50 to 1000 bp, preferably they have a size of 100 to 1000 bp.

In particular embodiments, the flanking regions of the recombinant poxvirus vector are flanking regions upstream and downstream of a deletion site of the MVA genome. In a specific embodiment, the flanking regions are flanking regions 50 to 1000 bp, preferably 100 to 1000 bp, upstream and downstream of Deletion sites I, II, III, IV, V, or VI of the MVA genome (MVA genome position: Del I: 7608/7609, Del II: 20718/20719; Del III: 149341/149342; Del IV: 170480; Del V: 19754/19755; Del VI 9831/9832). SEQ ID NOs: 2 -7 show the flanking regions of Deletion sites I-VI, respectively, centered around the Deletion site that may be used according to the present invention. As an example, SEQ ID NO: 2 is a sequence of 2002 bp, centered around Deletion site I of the MVA genome (position 7608/7609). Thus, positions 1001/1002 of SEQ ID NO: 2 correspond to positions 7608/7609 of the MVA genome. Accordingly, the flanking regions in one specific embodiment of the invention are 50 to 1000 bp upstream of position 1001 of SEQ ID NO: 2 and 50 to 1000 bp downstream of position 1002 of SEQ ID NO: 2.

In another specific embodiment, the flanking regions of the recombinant poxvirus vector are selected in a way that the insertion site will be positioned within a non-coding region of the poxvirus. Non-coding regions of poxviruses vary in length and may comprise from around 100 to more than 1000 bp. Each of the nucleotides of such a non-coding region may be selected as insertion site. The flanking regions according to the inventions are 50 to 1000 bp, preferably 100

to 1000 bp, upstream and downstream of this selected insertion site located within a non-coding region. If, for example, the non-coding region has a length of 1000 bp and the insertion site would be selected to be between positions 500 and 501 than the flanking regions according to the invention would be 50 to 1000 bp upstream from position 500 and 50 to 1000 bp downstream to position 501. In specific embodiments, the non-coding region is a non-coding region of MVA. In further specific embodiments, the non-coding region is the nucleotide sequence between any of the following genes of MVA (Gen Bank Accession # U94848): 001L-002L, 002L-003L, 005R-006L, 006L-007R, 007R-008L, 017L-018L, 018L-019L, 020L-021L, 023L-024L, 024L-025L, 025L-026L, 028R-029L, 030L-031L, 031L-032L, 032L-033L, 035L-036L, 036L-037L, 037L-038L, 039L-042L, 043L-044L, 044L-045L, 046L-047L, 049L-050L, 050L-051L, 051L-052L, 052R-053R, 053R-054R, 054R-055R, 055R-056L, 056L-057R, 061L-062L, 064L-065L, 065L-066L, 066L-067L, 077L-078R, 078R-079L, 080R-081L, 085R-086R, 086R-087R, 088R-089L, 089L-090R, 094L-095R, 096R-097R, 097R-098R, 101R-102R, 103R-104R, 105L-106R, 108L-109L, 109L-110L, 110L-111L, 113L-114L, 114L-115L, 115L-116R, 117L-118L, 118L-119R, 123L-124L, 124L-125L, 125L-126L, 133R-134R, 134R-135R, 137L-138L, 141L-142R, 143L-144R, 144R-145R, 145R-146R, 146R-147R, 147R-148R, 148R-149L, 152R-153L, 153L-154R, 154R-155R, 156R-157L, 157L-158R, 159R-160L, 160L-161R, 161R-162R, 165R-166R, 166R-167R, 170R-173R, 173R-174R, 174R-175R, 175R-176R, 176R-177R, 178R-179R, 179R-180R, 180R-181R, 183R-184R, 184R-185L, 185L-186R, 186R-187R, 187R-188R and/or 188R-189R.

In another specific embodiment, the flanking regions of the recombinant poxvirus vector are selected in a way that the insertion site will be positioned within a non-essential gene of the poxvirus. A gene is considered to be a non-essential gene if it is not required for the productive replication of a given poxvirus. In specific embodiments, the non-essential gene is selected from the group consisting of the Thymidine kinase gene (= Tk; Scheiflinger F. *et al.* "Evaluation of the thymidine kinase (tk) locus as an insertion site in the highly attenuated vaccinia MVA strain", Arch. Virol. 1996, 141(3-4): 663-9.), the hemagglutinin gene (= HA; Antoine G. *et al.* "Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes", Gene, 1996 Oct 24; 177(1-2): 43-6), the I4L coded ribonucleotide reductase (Howley PM *et al.* "A Vaccinia Virus transfer vector using a GUS reporter gene inserted into the I4L locus", Gene, 1996 Jun 26; 172(2): 233-7), the E3L (Langland JO & Jacobs BL, "The role of the PKR-inhibitory genes, E3L and K3L, in determining vaccinia virus host range", Virology, 2002 Jul 20; 299(1): 133-41), the K1L (Staib C. *et al.* "Recombinant

MVA and method for generation thereof" EP1594970, 2005 November 16) and the ATI gene (Wintersperger S. *et al.* "Vector for integration of heterologous sequences into poxviral genomes", 2002 Feb 20). In further specific embodiments, the non-essential gene is a gene from MVA and selected from 032L (Ribonucleotide reductase), 137L (function not known), 166R (Guanylate kinase fragment), 170 (function not known), 188R (function not known) and 189R (function not known).

In another embodiment of the invention, the poxvirus is a vaccinia virus. In a further embodiment, the poxvirus is MVA.

In a further aspect, the invention relates to a cell harboring the modified poxvirus vector of the present invention. Generally, every cell that is permissive for receiving the modified poxvirus vectors of the present invention is encompassed. Preferably, the cell is a eukaryotic cell. In a particular embodiment, the cell is a mammalian or an avian cell. In a specific embodiment, the cell is a chick embryo fibroblast (CEF) cell. In a further embodiment, the cell is an isolated human cell.

In a further aspect the invention is directed to the novel plasmids and vectors used for the generation of the modified poxvirus vector according to the invention. The present invention thus also encompasses a nucleic acid or its complement selected from the group consisting of SEQ ID NOs: 15-23 or a fragment or homologous nucleic acid thereto that shares at least 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 95%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.7%, 99.8%, 99.9% sequence identity, wherein the fragment has a length of at least 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 95%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 98.5%, 99%, 99.5%, 99.7%, 99.8%, 99.9% of the respective nucleic acid selected from the group consisting of SEQ ID NOs: 15-23.

Methods of the present invention

In a further aspect, the invention is directed to a method for generating a recombinant poxvirus, wherein a host cell permissible for the modified poxvirus vector of the present invention is transfected with said vector, and furthermore with a plasmid comprising heterologous genetic material of interest under conditions permitting homologous recombination between the vector and the plasmid within the host cell. Necessarily, both the modified poxvirus vector and the plasmid comprise flanking sequences that are similar enough to allow for such homologous recombination to occur. Furthermore, the modified poxvirus

vector carries the reporter gene and in certain embodiments the selection component as described above between said flanking regions, while the plasmid carries the heterologous genetic material of interest between said flanking regions. Accordingly, homologous recombination between the modified poxvirus vector and the plasmid produces a recombinant
5 vector of the invention through replacement of the reporter gene (and the selection component, if present) by the heterologous genetic material of interest.

In certain embodiments, the method utilizes a modified poxvirus vector of the invention comprising a selection component, and further comprises the additional step of inducing expression of the selection gene in the host cell. The induction of selection gene expression
10 depends on the inducible selection component that is employed. As an example and in a specific embodiment, the selection component comprises a selection gene that encodes the DNase protein which is expressed under the regulatory control of the bacterial inducible tetracycline repressor binding site/operator (TetO2) as described above. Induction is carried out by the addition of tetracycline, which binds to the tetracycline repressor that immediately detaches from
15 the binding site on the DNA and, this in turn allows expression of the DNase that inhibits virus replication. This induction results in a desirable selection of host cells comprising recombinant vector relative to host cells comprising modified poxvirus vector that does not comprise the gene insert of interest.

In a further embodiment, the method of the invention further comprises an additional
20 step of separating host cells comprising the recombinant vector from those host cells comprising non-recombinant modified poxvirus vector. In a particular embodiment, this separation is carried out by means of the gene product of the expressed reporter gene. In one embodiment, the reporter gene codes for a fluorescent protein and the separation is carried out by means of FACS and an attached cell sorter. In another embodiment, the reporter gene is an arbitrary
25 protein that is expressed on the cell surface and to which an antibody exists. Separation is then achieved by coating magnetic beads with said antibodies and contacting the cells with said magnetic beads in order to separate the two cell populations. In another embodiment, the antibodies are attached to a resin and used in a column to separate the two cell populations.

In yet another embodiment, the separated host cells comprising the recombinant vector
30 are used for at least one additional passage on fresh host cells. Accordingly, the number of cells harboring recombinant vector, and thus the number of generated recombinant poxvirus particles, is significantly increased. In particular embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9 or 10

further passages of the recombinant poxvirus are carried out. In a specific embodiment, 6 further passages of the recombinant poxvirus are carried out. In a further embodiment, the method includes the step of inducing expression of the selection gene.

In a further embodiment of the method of the present invention, the poxvirus is vaccinia virus or MVA.

The generated recombinant poxviruses can be isolated using any suitable technique known to the average skilled person, for example, by means of centrifugation.

Medical uses of the present invention

In a further aspect, the present invention is directed to the modified poxvirus according to the invention, the recombinant poxvirus according to the invention, or a cell comprising the modified or recombinant poxvirus according to the invention for use as a medicament. In a specific embodiment, the medicaments of the instant invention are advantageous for use as a therapeutic or prophylactic vaccine.

In another embodiment, the present invention is directed to the modified poxvirus according to the invention, the recombinant poxvirus according to the invention, or a cell comprising the modified or recombinant poxvirus according to the invention for use as a medicament for the treatment and/or prevention of an infection in a species selected from the group consisting of Influenza virus A, B, C, Hepatitis virus A, B, C, E, Human Immunodeficiency virus, Rubella virus, Mumps virus, Rabies virus, Human papilloma virus, Epstein Barr virus, Tickborne virus, Crimean Kongo Fever virus, Ebola virus, Nipah virus, Dengue virus, Chikungunya virus, Enterovirus, West Nile virus, Rift Valley Fever virus, Japanese encephalitis virus, Hantavirus, Rotavirus, SARS Coronavirus, Emerging viruses, Chlamydia trachomatis, Clostridium botulinum, Clostridium tetani, Bacillus anthracis, Legionella pneumophila, Neisseria meningitidis (Menigococcus), Yersinia pestis, Mycobacterium tuberculosis, Mycobacterium leprae, Salmonella typhi, Listeria monocytogenes, Vibrio cholerae, Haemophilus influenzae, Bordetella pertussis, Helicobacter pylori, Borrelia spp. (recurrentis, hispanica, parkeri, burgdorferi), Leptospira interrogans, Rickettsia spp., Coxiella burnettii, Mycoplasma pneumonia, Corynebacterium diphtheriae, Treponema pallidum, Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Entamoeba histolytica, Giardia intestinalis, Trypanosoma brucei, Leishmania spp., Histoplasma capsulatum, Aspergillus spp., Candida albicans, Cryptococcus neoformans, Pneumocystis carinii, Wuchereria bancrofti,

Schistosoma mansoni and/or Toxoplasma gondii. In further specific embodiments, the medicament is used for the treatment of a disease or the symptoms caused by any of these species. In particular embodiments, this disease is influenza, hepatitis, AIDS, mumps, rabies, encephalitis, stomach or duodenal ulcers, malaria, sleeping sickness, Lyme disease, reactive
5 arthritis, pneumonia, leprosy, diphtheria, candidiasis and/or toxoplasmosis.

In a further embodiment, the present invention is directed to the modified poxvirus according to the invention, the recombinant poxvirus according to the invention, or a cell comprising the modified or recombinant poxvirus according to the invention for use as a medicament for the treatment and/or prevention of cancer. In specific embodiments, the cancer
10 is selected from the group consisting of cervical carcinoma, melanoma, multiple myeloma, breast cancer, prostate cancer, follicular B-cell non-Hodgkins lymphoma and/or kidney cancer.

In one embodiment of the invention, the medicament is used to treat a patient in need of a vaccination. In another embodiment of the invention, the recombinant poxvirus of the invention or the cell comprising said recombinant poxvirus comprises at least one gene coding
15 for an antigen that is capable of inducing an immune response in a patient requiring a vaccination. In specific embodiments, the antigen is selected from the group consisting of an antigen of a heterologous virus, an antigen of a bacterium, an antigen of a prokaryote, an antigen of a fungus, and/or an antigen of a helminth. In particular embodiments, the antigen is an antigen of a species selected from the group consisting of Influenza virus A, B, C, Hepatitis virus
20 A, B, C, E, Human Immunodeficiency virus, Rubella virus, Mumps virus, Rabies virus, Human papilloma virus, Epstein Barr virus, Tickborne virus, Crimean Kongo Fever virus, Ebola virus, Nipah virus, Dengue virus, Chikungunya virus, Enterovirus, West Nile virus, Rift Valley Fever virus, Japanese encephalitis virus, Hantavirus, Rotavirus, SARS Coronavirus, Emerging viruses, Chlamydia trachomatis, Clostridium botulinum, Clostridium tetani, Bacillus anthracis, Legionella
25 pneumophila, Neisseria meningitidis (Menigococcus), Yersinia pestis, Mycobacterium tuberculosis, Mycobacterium leprae, Salmonella typhi, Listeria monocytogenes, Vibrio cholerae, Haemophilus influenzae, Bordetella pertussis, Helicobacter pylori, Borrelia spp. (recurrentis, hispanica, parkeri, burgdorferi), Leptospira interrogans, Rickettsia spp., Coxiella burnettii, Mycoplasma pneumonia, Corynebacterium diphtheriae, Treponema pallidum, Plasmodium
30 falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Entamoeba histolytica, Giardia intestinalis, Trypanosoma brucei, Leishmania spp., Histoplasma capsulatum, Aspergillus spp., Candida albicans, Cryptococcus neoformans, Pneumocystis carinii, Wuchereria bancrofti, Schistosoma mansoni and/or Toxoplasma gondii. In a further specific embodiment, the antigen

is a protein expressed by a cancer cell. In another specific embodiment, the antigen is a protein that is expressed by and displayed on the surface of a cancer cell.

In further specific embodiments, the recombinant poxvirus of the invention, or the cell comprising said recombinant poxvirus comprises an antigen expressed by a cancerous cell.

5 Examples of such an antigen expressed by a cancerous cell include, but are not limited to, alpha-fetoprotein (AFP); prostate specific antigen (PSA); carcinoembryonic antigen (CEA); disialosyl LEA antigen; Melan-A/MART-1; SART3; multidrug resistance-associated protein 3 (MRP3); polycomb group protein enhancer of zeste homolog 2 (EZH2); ALDH1; Her-2; Nectin-4; gp96

10 heat shock protein; gp100; tyrosinase; GM2; MAGE-A3; and NY-ESO-1. In a further embodiment the recombinant poxvirus of the invention or the cell comprising said recombinant poxvirus comprises a gene encoding an antibody. In specific embodiments, such antibodies are specific for cell markers expressed on tumor cells.

The medicaments of the present invention can be administered to a patient by means known to the skilled person. In some embodiments of the invention, the medicaments are

15 administered by injection.

EXAMPLES

For assessing the efficacy of the modified poxvirus vectors of the present invention, several foundational MVA vectors were created. These MVA vectors contain a fluorescence coding reporter gene under the control of a strong vaccinia virus promoter, and a selection component comprising a DNase selection gene under the control of a strong vaccinia virus promoter and the Tetracycline operator (TetO2) element, and the Tetracycline repressor (TetR) coding gene under the control of different promoters.

As shown in FIG. 1, the basic MVA genome was modified by the introduction of a reporter gene/selection component cassette into the insertion site (Deletion III or Del3) of the MVA genome: adjacent to the upstream flanking region of Deletion III (Flank1 Del3) a reporter gene, for example, a green fluorescence protein (GFP) gene was inserted under the control of a strong synthetic vaccinia virus promoter (Ps) as a reporter system. This reporter, for example, GFP, optimally allows the detection of the MVA that contains the cassette insert. Downstream of the reporter gene (adjacent to Flank2 Del3), a selection component was inserted into the genome. This consists of, for example, a DNase gene under the control of a strong synthetic vaccinia virus promoter (Ps), which is controlled by a bacterial operator element (TetO2) that interacts with a repressor (TetR). The repressor interaction is in turn controlled by tetracycline. In the absence of tetracycline, TetR binds to the TetO2 control sequence to thereby inhibit the expression of the DNase gene. However, in the presence of tetracycline, TetR is released from the TetO2 site to thereby allow expression of the DNase gene. The expressed DNase protein then inhibits the amplification of the viral DNA in the cytoplasm of the infected cell.

For the isolation of recombinant MVA comprising the heterologous genetic material of interest, the reporter gene/selection component cassette is deleted by means of homologous recombination. Subsequently, the resulting virus is passaged in the presence of tetracycline. Cells comprising non-recombinant modified MVA are detectable by fluorescence, and DNase expression inhibits the growth of MVA. Cells comprising recombinant MVA lack the reporter gene/selection component cassette, and therefore they do not exhibit fluorescence, and their MVA growth is not inhibited since DNase expression does not occur. The isolation of the recombinant and the non-recombinant MVA can be performed by separation of the fluorescing and the non-fluorescing cells.

A bacterial plasmid containing the flanking sequences of the MVA Deletion III is used (FIG. 2 and 3) as a shuttle for the insertion of the heterologous genetic material of interest into

the modified MVA vector. The plasmid further comprises a LacZ expression cassette flanked by a plurality of single cutter restriction sites. The heterologous genetic material of interest that is to be inserted in the modified MVA vector is cloned into the bacterial vector vEM07 (SEQ ID NO: 1) by means of one or more single cutter restriction sites. The LacZ gene is deleted and the plasmid containing the heterologous genetic material of interest can be detected by blue white selection using X-Gal. The resulting plasmid is then used for the insertion of the heterologous genetic material of interest in the modified MVA vector by means of homologous recombination.

10 **EXAMPLE 1: Cloning of the modified MVA vector selection component**

The reporter gene/selection component cassette was inserted into the MVA genome by homologous recombination. For this purpose, the single components of (i) reporter gene, (ii) Ps-TetO2-DNAse, and (iii) TetR were cloned in a stepwise fashion into a bacterial plasmid.

Cloning of the DNAse gene

15 The DNAse fragment (SpeI-Ps-TetO2-DNAse-SacI) was synthesized via a two-step PCR process and cloned in the vector vEM11 (SpeI/SacI) (SEQ ID NO: 15), resulting in the production of vEM12 (SEQ ID NO: 16).

For the first PCR step, the following oligonucleotides were used:

- 1) oEM167: TetO2-DNAse start; TCCCTATCAGTGATAGAGATCTCCCTATCAGTG
20 ATAGAGATATGAGGGGCATGAAGCTGCTG (SEQ ID NO: 8)
- 2) oEM168: DNase end – SacI; GAGCTCCTACTTCAGCATCACC (SEQ ID NO: 9)

The template for this PCR process was the plasmid pENTR-DNAse (Invitrogen ORF Clone collection, clone ID IOH23149) that contained a human DNase open reading frame. This first PCR step resulted in the fragment depicted in FIG. 4.

25 For the second stage of the PCR, the purified PCR product of the first PCR step, and following oligonucleotides were used:

- 1) oEM169: SpeI-Ps-TetO2; GACTAGTAAAAATTGAAATTTTATTTTTTTTTTTTGG
AATATAAATATCCCTATCAGTGATAGAG (SEQ ID NO: 10)
- 2) oEM168: DNase end – SacI; GAGCTCCTACTTCAGCATCACC (SEQ ID NO: 9)

This second PCR step resulted in the fragment shown in FIG. 5.

The resulting fragment was then cloned into the recombination vector vEM11 (FIG. 6) through use of the SpeI and SacI restriction sites. This cloning step resulted in vector vEM12 (FIG. 7).

5 Cloning of TetR

During the next cloning step, the TetR coding region was inserted into vector vEM12. The amount of expressed TetR is an important experimental parameter, as it has to be sufficiently expressed in order to cover all TetO2 sequences present in the cell (about 200 copies per cell), but should not be expressed in excess as this would require an excess of tetracycline in
10 order to induce the expression of the DNase. Therefore, the TetR expression was assessed under the control of three different promoters: the Ps (strong expression), the H5 (medium expression) and the p7.5 (low expression).

Cloning of Cloning of TetR with the Ps promoter

The Ps - Tetracycline repressor (TetR) cassette was synthesized by PCR. The template
15 was the pcDNA6/TR plasmid (Invitrogen), and the following oligonucleotides were used for the fusion of the Ps promoter and the amplification:

- 1) oEM163: SacI-Ps-TetRstart; GGGAGCTCAAAAATTGAAATTTTATTTTTTTTTTTT
TGGAATATAAATAATGTCTAGATTAGATAAAAAG (SEQ ID NO: 11)
- 2) oEM164: TetRend-NheI; GCTAGCTTAATAAGATCTGAATTCC (SEQ ID NO: 12)

20 The resulting PCR fragment (FIG. 8) was cloned using SacI and NheI in vector vEM12. This cloning step resulted in vector vEM31, SEQ ID NO: 17 (FIG. 9).

Cloning of TetR with the p7.5/H5 promoter

The p7.5 and H5 promoter are present in the plasmids pVIV06 (SEQ ID NO:18) and pVIV07 (SEQ ID NO: 19), and TetR was amplified for insertion into these vectors by PCR
25 using the following oligonucleotides:

- 1) oEM165: XhoI-TetRstart; CTCGAGATGTCTAGATTAGATAAAAAG (SEQ ID NO: 13)
- 2) oEM166: TetRend-ApaI; CCGGGCCCTTAATAAGATCTGAATTCC (SEQ ID NO: 14)

For appropriate fusion of the TetR coding region to the promoters, PCR fragments were cloned via *ApaI* and *XhoI* in vector pVIV06 (p7.5) and vector pVIV07 (H5).

The cloning of the TetR in pVIV06 resulted in vector pEM12 (7.5) (SEQ ID NO:20), and the cloning in pVIV07 resulted in vector pEM13 (H5) (SEQ ID NO: 21). The TetR expression cassettes were then extracted using *SacI* and *ApaI* and then cloned in vEM12. This resulted in vector vEM32 (7.5) (SEQ ID NO: 22) and vector vEM33 (H5) (SEQ ID NO: 23), which were identical to vector vEM31 (FIG. 9), except for the promoter that is used to express the TetR gene.

10 EXAMPLE 2: Cloning of the modified MVA vector

vEM31, 32 and 33 were each employed to create modified MVA vectors. For this purpose, CEF cells were infected with MVA (moi 0.05) and then transfected with vEM31, 32 or 33, individually. The MVA particles released by the infected CEF cells were then passaged three times using fresh CEF cells and under selective conditions (e.g. blasticidine containing medium; 5µg/ml), since cells containing recombinant MVA (i.e., the modified MVA vectors) are resistant to this antibiotic. The recombinant MVA particles were subsequently purified by plaque purification and using a FACS unit (fluorescence activated cell sorter).

Following amplification and characterization of the resulting modified MVA vectors resulting from recombination of MVA with vEM31 (mEM06, Ps-TetR), with vEM32 (mEM07, 7.5-TetR) and with vEM33 (mEM08, H5-TetR), the tetracycline/DNase system was used for testing.

EXAMPLE 3: Assessment of the tetracycline/DNase system

For testing the inducible reverse selection system, cells were infected with modified MVA viruses mEM06, 07 and 08 individually and incubated in tetracycline-containing medium. Different concentrations of tetracycline were used for analysis (0-500 µg/ml).

In order to assess the influence of the inducible DNase expression on the replication of unmodified MVA, MVA-infected cells were transfected with (vEM12) to facilitate the transient expression of DNase. Furthermore, the influence of tetracycline on the replication of unmodified MVA was analyzed by incubating MVA-infected cells with tetracycline only. Additionally, for monitoring the influence of tetracycline on the growth and status of cells, MVA

infected and uninfected CEF cells were incubated with the identical tetracycline concentrations used for testing of the modified virus vectors.

Briefly, CEF cells were infected with the modified MVA viruses mEM07 (7.5 = 7.5 promoter), mEM06 (Ps = Ps promoter), mEM08 (H5 = H5 promoter) and incubated with media containing different concentrations of tetracycline (Tet 0 = no tetracycline; Tet 25 = 25 µg tetracycline per ml). For controls, CEF cells were infected with unmodified MVA empty vector (MVA), and some MVA infected cells were also subsequently transfected with vEM12 (MVA + DNase). CEF cells infected with unmodified MVA empty vector were also incubated with tetracycline (MVA + Tet). After 48 hours of incubation at 37°C (5% CO₂), cells were harvested and the virus titer was determined. FIG. 10 provides a summary of the results obtained.

The unmodified MVA control (FIG. 10, MVA) resulted in a titer of $8.75E^{+06}$ TCID₅₀. Consequently, this is the titer that can be achieved by infection with unmodified MVA without any the presence of any inhibiting substances under these conditions. The expression of DNase resulted in a titer reduced by 2 log scales (FIG. 10, MVA + DNase, $6.56E^{+04}$ TCID₅₀/ml). However, since MVA infection was established by inoculation with $1.00 E^{+05}$ TCID₅₀/ml of unmodified MVA, this treatment did not result in an increase in amount of virus (i.e. no productive replication), and therefore DNase expression does inhibit viral replication effectively.

Cells infected with unmodified MVA and incubated with tetracycline demonstrated that tetracycline itself has an influence on MVA replication, likely due to a cytotoxic effect. If 25 – 50 µg tetracycline/ml were used, there was no detectable influence on MVA replication, while using a concentration of 100 µg/ml reduced the MVA replication by 1 log; furthermore, a concentration of 250 µg/ml inhibited the viral replication comparable to the observed effect of transiently expressed DNase (FIG. 10, MVA + Tet). Treatment of cells infected by modified MVA vectors mEM06 and mEM08 with 75 µg/ml tetracycline resulted in a reduced viral replication through induced DNase expression (FIG. 10, mEM06 and mEM08), and this was not due to direct tetracycline exclusively, as the viral titer was reduced even more significantly. This same effect can be observed using 100 µg tetracycline per ml. Concentrations of 150 µg/ml result in a pronounced cytotoxic effect of tetracycline such that the modified MVA viruses do not replicate at all. Accordingly, for further testing purposes, a narrow concentration window of 75 to 100 µg tetracycline per ml was used. Modified MVA vector mEM07 expresses TetR with a weak p7.5 promoter clearly produces an insufficient amount of TetR for inhibiting the DNase expression in any quantitative amount. Therefore, the viral replication is markedly reduced in all

experimental arrangements, and the virus itself is not applicable as a basic virus for the cloning of recombinant viruses with a single recombination vector.

EXAMPLE 4: Cloning of a recombinant poxvirus vector using the single recombination system

Since modified MVA viruses mEM06 and mEM08 demonstrated similar replication patterns, additional experiments were performed using mEM06.

Cells were infected with modified MVA virus mEM06 and transfected with vEM07 (FIG. 3). Due to homologous recombination, the LacZ gene was inserted in select modified MVA virus genomes and the reporter/selection cassette was deleted. The released virus particles were passaged on fresh CEF cells in medium containing tetracycline (100 µg/ml), and then passaged by serial dilution on cells without selective conditions. Infected cells were sorted using a FACS to select non-fluorescing cells. Once the settings for the sorting process were optimized, the sorted cells were homogenized and passaged again on CEF cells. The infected cells were sorted and the virus was amplified once more on fresh CEF cells. The cells were clearly infected and exhibited no fluorescence, meaning that they contained purified recombinant MVA virus. Recombinant virus purity was confirmed by a PCR procedure that amplified the insertion site of the modified MVA vector (FIG. 11). The PCR produced a 3.0 kb signal for the modified MVA vector, and a smaller 0.76 kb fragment for the recombinant MVA containing LacZ which had replaced the reporter/selection cassette by homologous recombination.

The recombinant MVA virus samples provided a clear signal at 0.76 kb (FIG. 11, rec MVA), but no signal for modified MVA (mEM06) or unmodified MVA (MVA). The recombination vector vEM07 also produced the expected signal of 0.76 kb (FIG. 11, vEM07). The modified MVA containing the selection/reporter cassette within the deletion site was used as a control, and this showed the expected signal of 3.0 kb (mEM06). The unmodified MVA vector also resulted in the expected signal of about 200 base pairs (MVA).

In order to confirm the robust results achieved by the present system, the cloning process was repeated using the procedures described above. A recombinant MVA was again readily isolated using the described single recombination system.

EXAMPLE 5: Cloning of a further recombinant poxvirus vector using the single recombination system

A recombinant poxvirus is cloned using the single recombination system according to the invention using a modified poxvirus vector comprising a reporter gene located between a pair of flanking sequences allowing for homologous recombination is used.

Cells are infected with the modified MVA virus and then transfected with vEM07 (FIG. 3). Due to homologous recombination, the LacZ gene is inserted in select modified MVA virus genomes and consequently the reporter cassette is deleted. The released virus particles are then passaged at least once on fresh CEF cells in medium containing tetracycline (100 µg/ml).

Infected cells are then sorted using FACS to select non-fluorescing cells as described above. After the infected cells have been sorted the virus is amplified once more on fresh CEF cells.

The recombinant virus purity is confirmed by a PCR procedure that amplifies the insertion site of the modified MVA vector (FIG. 11). The PCR produces a fragment for the recombinant MVA containing LacZ which has replaced the reporter cassette by homologous recombination.

SEQUENCE LISTING

<110> EMERGENT PRODUCT DEVELOPMENT GERMANY GMBH
 LEYRER, Sonja
 5 MAYER, Katja

<120> SINGLE RECOMBINATION SYSTEM AND METHODS OF USE

<130> 145-009P

10 <150> EP 08022296.1
 <151> 2008-12-22

15 <150> US 61/139,991
 <151> 2008-12-22

<160> 23

20 <170> PatentIn version 3.3

 <210> 1
 <211> 4335
 <212> DNA
 <213> Artificial

25 <220>
 <223> Recombination vector vEM07

<400> 1

30 ctaaattgta agcgttaata ttttggtaaa attcgcgtta aatttttgtt aaatcagctc 60
 atatttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaaag aatagaccga 120
 gataggggttg agtgttggtc cagtttgga caagagtcca ctattaaaga acgtggactc 180
 35 caacgtcaaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
 ctaatacaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaaggagag 300
 40 cccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatgggtg 360
 tattgtatac tgtctaaacg cgtagtaaaa acatggcgag gaaataaatc atataaaaaa 420
 tgatttcatg attaaaccat gttgtgaaaa agtcaagaac gttcacattg gcggacaatc 480
 45 taaaaacaat acagtgattg cagatttgcc atatatggat aatgcggtat ccgatgtatg 540
 caattcactg tataaaaaaga atgtatcaag aatatccaga tttgctaatt tgataaagat 600
 50 agatgacgat gacaagactc ctactggtgt atataattat tttaaaccta aagatgccat 660
 tctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga 720
 taaagcgtgt gcgtgtatag agttaaattc atataaagta gccattcttc ccatggatgt 780
 55 ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga 840
 tgccggcacct ctcttaagaa gtgtaaccga taataatggt attatatcta gacaaccagc 900
 60 tctacatgac gagcttccga gttccaattg gttcaagttt tacataagta taaagtccga 960

	ctattgttct atattatata tggttgttga tggatctgtg atgcatgcaa tagctgataa	1020
5	tagaacttac gcaaataatta gcaaaaatat attagacaat actacaatta acgatgagtg	1080
	tagatgctgt tattttgaac cacagattag gattcttgat agagatgaga tgctcaatgg	1140
	atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg taggcgaatt	1200
10	tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc ttccggtggc	1260
	tggcacatcg gtggcgcccg ctctagaact agtggatccc ccgggctgca ggaagacatg	1320
15	catcgagatc tggcgccgtt tattcgccat tcaggctgcg caactgttgg gaagggcgat	1380
	cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg gggatgtgct gcaaggcgat	1440
	taagttgggt aacgccaggg ttttcccagt cacgacgttg taaaacgacg gccagtgaat	1500
20	tgtaatacga ctactatag ggcgaattgg agctccatcg cggcggcggg cgttcgagaa	1560
	cgagtgggtc ccctgggcta caggagttcg agatcaaact tattgatacc gttgatcttg	1620
25	aggggggtcc cggtaccagc ttttgttccc tttagtgagg gttaatttcg agcttggcgt	1680
	aatcatggtc atagctgttt cctgtgtgaa attgttatcc gtcacaatt ccacacaaca	1740
	tacgagccgg aagcataaag tgtaaagcct ggggtgccta atgagtgagc taactcacat	1800
30	taattgcgtt gcgcaaactt aattaacact tccgtggcta gcgaattcga tatcaagctt	1860
	atcgataccg tcgacctcga gggggggccc ggaaagtttt ataggtagtt gatagaacaa	1920
35	aatacataat tttgtaaaaa taaatcactt tttatactaa tatgacacga ttaccaatac	1980
	ttttgttact aatatcatta gtatacgcta caccttttcc tcagacatct aaaaaaatag	2040
	gtgatgatgc aactttatca tgtaatcgaa ataatacaaa tgactacgtt gttatgagtg	2100
40	cttgggtataa ggagcccaat tccattattc ttttagctgc taaaagcgac gtcttgtatt	2160
	ttgataatta taccaaggat aaaatatctt acgactctcc atacgatgat ctagttacaa	2220
45	ctatcacaat taaatcattg actgctagag atgccggtac ttatgtatgt gcattcttta	2280
	tgacatcgcc taaaaatgac actgataaag tagattatga agaatactcc acagagttga	2340
	ttgtaaatac agatagtga tgcactatag acataatact atctggatct acacattcac	2400
50	cggaaactag ggcttcctcg ctactgact cgctgcgctc ggtcgttcgg ctgcggcgag	2460
	cggatatcagc tcaactcaaag gcggtaatac ggttatccac agaatacagg gataacgcag	2520
55	gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa ccgtaaaaag gccgcgttgc	2580
	tggcgttttt ccataggctc cgccccctg acgagcatca caaaaatcga cgctcaagtc	2640
	agaggtggcg aaacccgaca ggactataaa gataaccaggc gtttccccct ggaagctccc	2700
60	tcgtgcgctc tcctgttcog accctgccgc ttaccggata cctgtccgoc tttctccctt	2760
	cgggaagcgt ggcgctttct catagctcac gctgtaggta tctcagttcg gtgtaggctg	2820
65	ttcgctccaa gctgggctgt gtgcacgaac cccccgttca gcccgaccgc tgcgccttat	2880
	cgggtaacta tcgtcttgag tocaaccggg taagacacga cttatogcca ctggcagcag	2940

ccactggtaa caggattagc agagcgaggt atgtaggcgg tgctacagag ttcttgaagt 3000
 5 ggtggcctaa ctacggctac actagaagga cagtatttgg tatctgcgct ctgctgaagc 3060
 cagttacctt cggaaaaaga gttggtagct cttgatccgg caaacaacc accgctggta 3120
 gcggtgggtt ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag 3180
 10 atcctttgat cttttctacg ggggtctgacg ctcagtggaa cgaaaactca cgtaaggga 3240
 ttttggcat gagattatca aaaaggatct tcacctagat ctttttaaataaaaaatgaa 3300
 15 gttttaaatc aatctaaagt atatatgagt aaacttggtc tgacagttac caatgcttaa 3360
 tcagtgaggg acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc 3420
 ccgtcgtgta gataactacg atacgggagg gcttaccatc tggccccagt gctgcaatga 3480
 20 taccgcgaga ccacgctca ccggctccag atttatcagc aataaaccag ccagccggaa 3540
 gggccgagcg cagaagtggc cctgcaactt tatccgcctc catccagtct attaattggt 3600
 25 gccgggaagc tagagtaagt agttcgccag ttaatagttt gcgcaacggt gttgccattg 3660
 ctacaggcat cgtgggtgtc cgtcgtcgtc ttggtatggc ttcattcagc tccggttccc 3720
 aacgatcaag gcgagttaca tgatcccca tgttgtgcaa aaaagcgggt agtccttcg 3780
 30 gtccctcgat cgttgtcaga agtaagttgg ccgcagtgtt atcactcatg gttatggcag 3840
 cactgcataa ttctcttact gtcatgccat ccgtaagatg cttttctgtg actggtgagt 3900
 actcaaccaa gtcattctga gaatagtgtg tgccggcgacc gagttgctct tgcéccggcgt 3960
 35 caatacggga taataccgcg ccacatagca gaactttaaa agtgctcatc attggaaaac 4020
 gttcttcggg gcgaaaactc tcaaggatct tacgctgtt gagatccagt tcgatgtaac 4080
 40 ccactcgtgc acccaactga tcttcagcat cttttacttt caccagcgtt tctgggtgag 4140
 caaaaacagg aaggcaaaat gccgcaaaa aggggaataag ggcgacacgg aaatgttgaa 4200
 tactcatact cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga 4260
 45 gcggatacat atttgaatgt atttagaaaa ataaacaaat aggggttccg cgcacatttc 4320
 ccgaaaagt gccac 4335
 50
 <210> 2
 <211> 2002
 <212> DNA
 <213> Vaccinia virus strain Ankara
 55
 <400> 2
 gtggtaggag gatctacatc ctgactgat tccactcgg gatctggatc tgactcggac 60
 tctgtaattt ccgttacgga ttggcaaatc ttatcatcgg tcggtgtttg gtcttgcttt 120

	gtgactttga taataacatc gattcccata tgatgtttgt tttcttcttc cgtacacgat	180
5	gaggatgatt gctgaagact ggcaggcaca tgcattgccag tacgatatat tgtttcatga	240
	ttgctattga ttgagtactg ttctttatga ttctacttcc ttaccgtgca ataaattaga	300
	atatatcttc tactttttacg agaaattaat tattgtatct atgggtgaaa aacttactat	360
10	aaaaagcggg tgggttttga attagtgatc agtttatgta tatcgcaact accgggcata	420
	tggctacatt acccatatga taagagattg tatcagtttc gtagtcttga gtattggtat	480
15	tactatatag tatatagatg tcgacgctag agttactgtc tccgaatgcg gcatgatagt	540
	atcattcttt gctttcgtta actgttttga ggaagaatct ttgttattgc atttaacttc	600
	gaaattcaga gtgcacacct ttctcctgta aagaaacctg aagtcgctac cttattaaga	660
20	agacgggata gcagtcctta tgattcatag taatagttag ttccgacgtt gagatggatt	720
	cgctgagacc ggtagtggtc gtccgagtac acgatgtgtc gttaactgga tacagggtta	780
25	ttccacatc gatatagtta aaggatcttc tgggtacggg ttcgcatcta tctgcggaag	840
	agacgggtgtg agaatatgtt ccgagaccac acggagaaca gatgacgtct ccggatactc	900
	cgtatcctat tccacatctt gtttgggaaa cacatgcctt gcatccatga tcgggagagc	960
30	attcacagat tctattgtga gtcgtgttac acgatcgcgt cgacattgtt gacagaaacg	1020
	tgaccttcac tcttaccgtc gtccataaat acgttaggta tgtaccacat actgtcgcga	1080
35	acgatcgcgt catctcataa tgatttactt tttcataaatt aaagatgtga aagaaaaccg	1140
	aacaatatat ttttttagta atgtttatgc gagacatata aaataaactc cgtgtttatg	1200
	atgccggtaa atgtttttat catcttggac ggaatcgatt ttgtaatatg ccatggaaac	1260
40	aggacattat cactccatga taaattatct aatggagtcg atcctctcat tgttctttgc	1320
	gtatctcaat ctgtggcgtt tgcttcgttt aaataatata tcaaacatgg agacgcctga	1380
45	tatgtaggca ttcttcattc tattaatgtc tgctctatag cgctttagtt ccttatgacg	1440
	accggcgata tcatacttac tttagaagga aaatcatcat ctaggattaa ggcgtatctg	1500
	atacaggcga ataatggttc aggatataga tagcgtatat ctctattaaa tgcgtcaatc	1560
50	atagtctcta gagtgggatg gtaactcagt aataaatcaa ctagcttctc tttggtaact	1620
	gcttttctgg atggccgtat tgattatcga gcgtgacact cgctccatat tccaataacc	1680
55	gctttgcaaa ttgtatatta ttgacatcga ccgcgtaata tagtagagtt atcgatcata	1740
	tctatatcat ccatgtactt gcttagtata tcaaatacat cttcataaca gtgatacccg	1800
	caattattaa atctcgataa tatcagaccg tacatacata gacggccatt gttagatatg	1860

tgatttacag cgcggtgtcc atattttcca cgataaacct tacgacgttt acatcgacga 1920
 gattattatt aacaaagttg ttgtccgtcg tcttatccaa catgcattga atgataggta 1980
 5 tacttaaccat atcgccgtaa tg 2002

 <210> 3
 <211> 2002
 10 <212> DNA
 <213> Vaccinia virus strain Ankara

 <400> 3
 15 gtaagcattg totgtattct ggagctatct tctctatctc taatttctga acgttcacca 60
 atgtctctag ccactttggc actaatagcg atcattcgct tagcgtcttc tatattatta 120
 actggttgat tcaatctatc tagcaatgga cgcgcggaca gcgtcattct catgttctta 180
 20 atcaatgtac atacatcgcc gtcactctacc aattcatcca acaacataag ctttttaaaa 240
 tcatcattat aataggtttg atcgttgtca tttctccaaa gaatatatct aataagtaga 300
 gtcctcatga ttagttaaca actatTTTTT atgttaaadc aattagtaca ccgctatggt 360
 25 taatacttat tcatatttta gtttttagga ttgagaatca atacaaaaat taatgcatca 420
 ttaatttttag aaatacttag tttccacgta gtcaatgaaa ctttgaact catcgtagag 480
 30 gacgttctcg tacaggacgt aactataaac cggtttatat ttgttcaaga tagatacaaa 540
 tccgataact ttttttacga attctacggg atccacttta aaagtgtcat accgggttct 600
 ttttattttt ttaaacagat taatgggtgt atgttgatta ggtcttttac gaatttgata 660
 35 tagaatagcg ttacatatt ctccataatg gtcaatcgcc atttggtcgt atgtcataaa 720
 ttctttaatt atatgacact gtgtattatt tagttcatcc ttgttcatca ttaggaatct 780
 40 atccaatatg gcaattatac tagaactata ggtgcgttgt atacacatat tgatgtgtct 840
 gtttatacaa tccatgctac taccttcggg taaaattgta gcatcatata ccatttctag 900
 tacttttaggt tcattgttat ccattgcaga ggacgtcatg atcgcatcct aaaaaaatat 960
 45 attattttta tgttattttg ttaaaaataa tcatcgaata cgaatcatcc agtccactga 1020
 atagcaaaat ctttactatt ttggtatctt ccaatgtggc tgcttgatgt aatggaaatt 1080
 50 cattctctag aagatttttc aatgctccag cgttcaacaa cgtacatact agacgcacgt 1140
 tattatcagc tattgcataa tacaaggcac tatgtccatg gacatccgcc ttaaatgcat 1200
 ctttgctaga gagaaagctt ttcagctgct tagacttcca agtattaatt cgtgacagat 1260
 55 ccattgtctga aacgagacgc taattagtgt ataatttttg tcatattgca ccagaattaa 1320
 taatatctct aatagatctg attagtagat acatggctat cgcaaaacaa catatacaca 1380

	tttaataaaaa ataatatatta ttaagaaaaat tcagattttca cgtaccccatc aatataaata	1440
	aaataatgat tccttacacc gtacccatat taaggagatt ctaccttacc cataaacaat	1500
5	ataaatccag taatatcatg tctgatgatg aacacaaatg gtgtattaaa ttccagtttt	1560
	tcaggagatg atctcgccgt agctaccata atagtagatg cctctgctac agttccttgt	1620
10	tcgtcgacat ctatctttgc attctgaaac attttataaa tatataatgg gtccttagtc	1680
	atatgtttaa acgacgcatt atctggatta aacatactag gagccatcat ttcggctatc	1740
	gacttaatat cctctttatt ttcgatagaa aatttaggga gtttaagatt gtacacttta	1800
15	ttccctaatt gaaacgacca atagtctaata tttgcagccg taatagaatc tgtgaaatgg	1860
	gtcatattat cacctattgc caggtacata ctaatattag catccttata cggaaggcgt	1920
	accatgtcat attctttgtc atcgattgtg attgtatttc cttgcaattt agtaactacg	1980
20	ttcatcatgg gaaccgtttt cg	2002
	<210> 4	
25	<211> 2001	
	<212> DNA	
	<213> Vaccinia virus strain Ankara	
	<400> 4	
30	tactcatcta aacgattttag taaacttgac taaatcttaa tagttacata caaattaaaa	60
	taacactatt tagttgggtg tgcgccatgga tgggtgttatt gtatactgtc taaacgcgtt	120
	agtaaaacat ggcgaggaaa taaatcatat aaaaaatgat ttcattgatta aaccatgttg	180
35	tgaaaaagtc aagaacgttc acattggcgg acaatctaaa aacaatacag tgattgcaga	240
	tttgccatat atggataatg cggatatccga tgtatgcaat tcaactgtata aaaagaatgt	300
40	atcaagaata tccagatttg ctaatttgat aaagatagat gacgatgaca agactcctac	360
	tgggtgtatat aattatttta aacctaaaga tgccattcct gttattatat ccataggaaa	420
	ggatagagat gtttgtgaac tattaatctc atctgataaa gcgtgtgcgt gtatagagtt	480
45	aaattcatat aaagtagcca ttcttcccat ggatgtttcc ttttttacca aaggaaatgc	540
	atcattgatt attctcctgt ttgatttctc tatcgatgcg gcacctctct taagaagtgt	600
50	aaccgataat aatgttatta tatctagaca ccagcgtcta catgacgagc ttccgagttc	660
	caattgggtc aagttttaca taagtataaa gtccgactat tgttctatat tatatatggg	720
	tgttgatgga tctgtgatgc atgcaatagc tgataataga acttacgcaa atattagcaa	780
55	aaatatatta gacaatacta caattaacga tgagtgtaga tgctgttatt ttgaaccaca	840
	gattaggatt cttgatagag atgagatgct caatggatca tcgtgtgata tgaacagaca	900

	ttgtattatg atgaatttac ctgatgtagg cgaatttgga tctagtatgt tggggaaata	960
	tgaacctgac atgattaaga ttgctctttc ggtggctggt atttggaag ttttataggt	1020
5	agttgataga acaaaatata taattttgta aaaataaatc actttttata ctaatatgac	1080
	acgattacca atacttttgt tactaatatc attagtatac gctacacctt ttcctcagac	1140
10	atctaaaaaa ataggtgatg atgcaacttt atcatgtaat cgaaataata caaatgacta	1200
	cgttgttatg agtgcttggt ataaggagcc caattccatt attcttttag ctgctaaaag	1260
	cgacgtcttg tattttgata attataccaa ggataaaata tcttacgact ctccatacga	1320
15	tgatctagtt acaactatca caattaaatc attgactgct agagatgccg gtacttatgt	1380
	atgtgcattc tttatgacat cgctacaaa tgacactgat aaagtagatt atgaagaata	1440
20	ctccacagag ttgattgtaa atacagatag tgaatcgact atagacataa tactatctgg	1500
	atctacacat tcaccggaaa ctagttctga gaaacctgat tatatagata attctaattg	1560
	ctcgtcggta ttcgaaatcg cgactccgga accaattact gataatgtag aagatcatac	1620
25	agacaccgtc acatacacta gtgatagcat taatacagta agtgcacat ctggagaatc	1680
	cacaacagac gagactccgg aaccaattac tgataaagaa gaagatcata cagttacaga	1740
30	cactgtctca tacactacag taagtacatc atctggaatt gtcactacta aatcaaccac	1800
	cgatgatgcg gatctttatg atacgtacaa tgataatgat acagtaccat caactactgt	1860
	aggcggtagt acaacctcta ttagcaatta taaaaccaag gactttgtag aaatatttgg	1920
35	tattaccgca ttaattatat tgtcggccgt ggcaatattc tgtattacat attatatata	1980
	taataaacgt tcacgtaaat a	2001
40	<210> 5	
	<211> 1996	
	<212> DNA	
	<213> Vaccinia virus strain Ankara	
45	<400> 5	
	ggcgatatgg taagtatacc tatcattcaa tgcattgttg ataagacgac ggacaacaac	60
	tttgtaata ataattctgt cgatgtaaac gtcgtaaggt ttatcgtgga aaatatggac	120
50	acgcggctgt aaatcacata tctaacaatg gccgtctatg tatgtacggt ctgatattat	180
	cgagatttaa taattgcggg tatcactgtt atgaagatgt atttgatata ctaagcaagt	240
55	acatggatga tatagatatg atcgataact ctactatatt acgcggtcga tgtcaataat	300
	atacaatttg caaagcgggt attggaatat ggagcgagtg tcacgctcga taatcaatac	360
	ggccatccag aaaagcagtt accaaagaga agctagttaga ttattactg agttaccatc	420

	ccactctaga gactatgatt gacgcattta atagagatat acgctatcta tatectgaac	480
	cattattcgc ctgtatcaga tacgccttaa tcttagatga tgattttcct tctaaagtaa	540
5	gtatgatatc gccggtcgtc ataaggaact aaagcgctat agagcagaca ttaatagaat	600
	gaagaatgcc tacatatcag gcgtctccat gtttgatata ttatttaaac gaagcaaacg	660
10	ccacagattg agatacgcaa agaacaatga gaggatcgac tccattaaat aatttatcat	720
	ggagtgataa tgtcctgttt ccatggcata ttacaaaatc gattccgtcc aagatgataa	780
	aaacatttac cggcatcata aacacggagt ttattttata tgtctcgcat aaacattact	840
15	aaaaaaatat attgttcggt tttctttcac atctttaatt atgaaaaagt aaatcattat	900
	gagatggacg catcgttcgc gacagtatgt ggtacatacc taacgtatct atggacgacg	960
20	gtaagaatga aggtcacgtt tctgtcaaca atgtcgacgc gatcgtgtaa cagcactcac	1020
	aatagaatct gtgaatgctc tcccgatcat ggatgcaagg catgtgtttc ccaaacaaaa	1080
	tgtggaatag gatacggagt atccggagac gtcactctgt ctcogtgtgg tctcggaaca	1140
25	tattctcaca ccgtctcttc cgcagataaa tgcgaaccgc taccagaaa tacctttaac	1200
	tatatcgatg tggaaattaa cctgtatcca gttaacgaca catcgtgtac tcggacgacc	1260
30	actaccggtc tcagcgaatc catctcaacg tcggaactaa ctattactat gaatcataaa	1320
	gactgcgatc ccgtcttctt aataaggtag cgacttcagg tttctttaca ggagaaaggt	1380
	gtgcactctg aatttcgaga ttaaatgcaa taacaaagat tcttcctcca aacagttaac	1440
35	gaaagcaaag aatgatacta tcatgccgca ttcggagaca gtaactctag cgtcgacatc	1500
	tatatactat atagtaatac caatactcaa gactacgaaa ctgatacaat ctcttatcat	1560
40	gtgggtaatg tagccatag cccggtagtt gcgatataca taaactgatc actaattcca	1620
	aaccaccccg ctttttatag taagtttttc acccataaat acaataatta atttctcgta	1680
	aaagtagaaa atatattcta atttattgca cggttaaggaa gtagaatcat aaagaacagt	1740
45	actcaatcaa tagcaatcat gaaacaatat atcgtactgg catgcatgtg cctgccagtc	1800
	ttcagcaatc atcctcatcg tgtacggaag aagaaaacaa acatcatatg ggaatcgatg	1860
50	ttattatcaa agtcacaaaag caagacaaaa caccgaccga tgataagatt tgccaatccg	1920
	taacggaaat tacagagtcc gagtcagatc cagatcccg ggtggaatca gtcgaggatg	1980
	tagatcctcc taccac	1996
55		
	<210> 6	
	<211> 2002	
	<212> DNA	
	<213> Vaccinia virus strain Ankara	

	<400> 6		
	aaataatttt tagtttgcac ccgtagttat cccctttatg taactgtaaa tttctcaacg	60	
5	cgatatctcc attaataatg atgtcgaatt cgtgctgtat acccatactg aatggatgaa	120	
	cgaataccga cggcgttaat agtaatttac tttttcatct ttacatattg ggtactagtt	180	
10	ttactatcat aagttttataa attccacaag ctactatgga ataagccaac catcttagta	240	
	taacacacat gtcttaaagt ttattaatta attacatggt gttttatata tcgctacgaa	300	
	tttaaacaga gaaatcagtt taggaaaaaa aaatatctat ctacatcatc acgtctctgt	360	
15	attctacgat agagtgtctac tttaagatga gacatatccg tgtcatcaaa aatatactcc	420	
	attaaaatga ttattccggc agcgaacttg atattggata tatcacaacc tttgttaata	480	
20	tctacgacaa tagacagcag tcccatgggt ccataaacag tgagtttata tttctttgaa	540	
	gagatatttt gtagagatct tataaaactg tcgaatgaca tcgcatttat atcttttagct	600	
	aaatcgtata tgttaccatc gtaatatcta accgcgtcta tcttaaactg ttccatcgt	660	
25	ttaaagacgt ttccgataga tggctctcatt tcatcagtc aactgagcca acaaatataa	720	
	tcgtgtataa catctttgat agaatcagac tctaaagaaa acgaatcggc tttattatac	780	
30	gcattcatga taaacttaat gaaaaatgtt tttcgttggt taagttggat gaatagtatg	840	
	tcttaataat tggtattatt tcattaatta atatttagta acgagtacac tctataaaaa	900	
	cgagaatgac ataactagtt atcaaagtgt ctaggacgcg taattttcat atggtataga	960	
35	tcctgtaagc attgtctgta ttctggagct attttctcta tatctaattt ctgaacgttc	1020	
	accaatgtct ctagccactt tggcactaat agcgatcatt cgcttagcgt cttctatatt	1080	
40	attaactggt tgattcaatc tatctagcaa tggaccgtcg gacagcgtca ttctcatggt	1140	
	cttaataaat gtacatacat cgccgtcatc taccaattca tccaacaaca taagcttttt	1200	
	aaaatcatca ttataatagg ttgatcgtt gtcattttctc caaagaatat atctaataag	1260	
45	tagagtcttc atgattagtt aacaactatt ttttatgtta aatcaattag tacaccgcta	1320	
	tgtttaatac ttattcatat tttagttttt aggattgaga atcaatacaa aaattaatgc	1380	
50	atcattaatt ttagaaatac ttagttttcca cgtagtcaat gaaacatttg aactcatcgt	1440	
	acaggacgtt ctgctacagg acgtaactat aaaccgggtt atatttggtc aagatagata	1500	
	caaatccgat aacttttttt acgaattcta cgggatccac tttaaaagtg tcataccggg	1560	
55	ttcttttttat ttttttaaac agattaatgg tgtgatgttg attaggtctt ttacgaattt	1620	
	gatatagaat agcgtttaca tattctccat aatgggtcaat cgcattttgt tcgtatgtca	1680	
	taaattcttt aattatatga cactgtgtat tatttagttc atcttggttc atcattagga	1740	

atctatccaa tatggcaatt atactagaac tataggtgog ttgtatacac atattgatgt 1800
 gtctgtttat acaatccatg ctactacott cgggtaaaat tgtagcatca tataaccattt 1860
 5 ctagtacttt aggttcattg ttatccattg cagaggacgt catgatcgca tcctaaaaaa 1920
 atatattatt tttatgttat tttgttaaaa ataatcatcg aatacgaatc atccagtcca 1980
 10 ctgaatagca aaatctttac ta 2002

 <210> 7
 <211> 2002
 15 <212> DNA
 <213> Vaccinia virus strain Ankara

 <400> 7
 20 ctaatatagg gctgacgtcg tatactctac acgtgtccac atcctttatt aataatctct 60
 atatctatgg ttgagcaaga ccagtagtat tggatggaaa cattgttatc gatcaaacat 120
 ttaattacat ccttggatag agattctcta tgagacgata tatagtaatg aagagagttc 180
 25 ttacacatat cactgttgta catacaggta cgaaatacgt aaccggtgct gtaacattct 240
 gatttaagaa gccatagcaa tacttctggt ctcgattag gcgtcgttac gtatatatcc 300
 30 accaatccga gaccattgat tgcataattc gtattcttgg acggacgtat ccgtttatcc 360
 acaattagggt attttagcag acgtaagtcg aaatcattta tattcgactt gagttcgtta 420
 gaggaattcg aatagctgga tatcagtaga tgcacaatct gagattttac gtatctatgc 480
 35 ttactgtatg ctctagcgg agttaatcct tcgttgtttc tacaaagtct ctcgactccg 540
 cgagagagta acagtcgaac aatcttaatg tctgtatcgc atttattgga gacgtaacaa 600
 tgtagcgcac tgtttcctcg tctatctata tgttttgata agttgtgaca cgtttcaatt 660
 40 tctagtttta tttttttgta cgtcacatct tcatccagta gacgacatag aatacatgtg 720
 caatccatag ctattctggt gctaattatt cctcataaga tgataaaaag tgtagtgaga 780
 45 gagcatgaag gagatttagt atttagcagt gcggatatga tccaagaggg tgagatagtc 840
 gttctcgttc agaatctttc gcagcataag tagtatgtcg atatacttat cgttgaagac 900
 tcttcagag acgatagctg attgagtaca aagtccaatg attgcacgaa gttcttcggc 960
 50 ggttttcatg gagtcatttc tgatgaaaca tttaatgac taaatttcag tttatgtttg 1020
 taccocgtat tcatacttaa caaattggta ttacatacca ttaataatgc aagcataaaa 1080
 55 aatcgttagt agatgtttct aaatataggt tccgtaagca aagaatataa gaatgaagcg 1140
 gtaatgataa aatcaatcgt tatctaaaaa gatcatactc atttatctta tctattata 1200
 ttaacacata catttttaac agcaacacat tcaatattgt attgttattt ttatattatt 1260

tacacaatta acaatatatt attagtttat attactgaat taataatata aaattcccaa 1320
 5 tcttgtcata aacacacact gagaaacagc ataaacacaa aatccatcaa aaatgttgat 1380
 aaattatctg atgttgttgt tcgctgctat gataatcaga tcattogccg atagtggtaa 1440
 cgctatogaa acgacatcgc cagaaattac aaacgctaca acagatattc cagctatcag 1500
 10 attatgcggt ccagagggag atggatattg tttacacggt gactgtatcc acgctagaga 1560
 tatcgacggt atgtattgta gatgctctca tggttataca ggcattagat gtcagcatgt 1620
 agtattagta gactatcaac gttcagaaaa accaaacact acaacgtcat atatcccatc 1680
 15 tcccggtatt atgcttgtat tagtaggcat tattattatt acgtgttgtc tattatctgt 1740
 ttataggttc actcgacgaa ctaaaactacc tatacaagat atggttgtgc cataatTTTT 1800
 20 ataaatTTTT tttatgagta tttttacaaa aatgtataaa gtgtatgtct tatgtatatt 1860
 tataaaaatg ctaaatatgc gatgtatcta tgttatttgt atttatctaa acaatacctc 1920
 25 tacctctaga tattatacaa aaatttttta tttcggcata ttaaagtaaa atctagttac 1980
 cttgaaaatg aatacagtggt gt 2002

30 <210> 8
 <211> 62
 <212> DNA
 <213> artificial

35 <220>
 <223> Primer oEM167

<400> 8
 tccctatcag tgatagagat ctccctatca gtgatagaga tatgaggggc atgaagctgc 60

40 tg 62

45 <210> 9
 <211> 22
 <212> DNA
 <213> artificial

50 <220>
 <223> Primer oEM168

<400> 9
 gagctcctac ttcagcatca cc 22

55 <210> 10
 <211> 65
 <212> DNA
 <213> artificial

<220>
 <223> Primer oEM169

 <400> 10
 5 gactagtaaa aattgaaatt ttattttttt tttttggaat ataaatatcc ctatcagtga 60
 tagag 65

 10 <210> 11
 <211> 68
 <212> DNA
 <213> artificial

 15 <220>
 <223> Primer oEM163

 <400> 11
 20 gggagctcaa aaattgaaat tttatttttt ttttttgga tataaataat gtctagatta 60
 gataaaaag 68

 25 <210> 12
 <211> 25
 <212> DNA
 <213> artificial

 30 <220>
 <223> Primer oEM164

 <400> 12
 gctagcttaa taagatctga attcc 25

 35 <210> 13
 <211> 26
 <212> DNA
 <213> artificial
 40
 <220>
 <223> Primer oEM165

 <400> 13
 45 ctcgagatgt ctagattaga taaaag 26

 50 <210> 14
 <211> 27
 <212> DNA
 <213> artificial

 55 <220>
 <223> Primer oEM166

 <400> 14
 ccgggccctt aataagatct gaattcc 27

```

    <210> 15
    <211> 5687
    <212> DNA
    <213> artificial
5
    <220>
    <223> vector vEM11

10
    <220>
    <221> misc_feature
    <222> (338)..(1263)
    <223> Flank1 Del3

15
    <220>
    <221> gene
    <222> (1283)..(1962)
    <223> Amazi green (C)

20
    <220>
    <221> misc_feature
    <222> (2002)..(1963)
    <223> Ps

25
    <220>
    <221> misc_feature
    <222> (2009)..(2048)
    <223> Ps

30
    <220>
    <221> gene
    <222> (2049)..(2447)
    <223> BsdR

35
    <220>
    <221> gene
    <222> (2463)..(2653)
    <223> MAV164R (putative 34.9k protein)

40
    <220>
    <221> misc_feature
    <222> (2463)..(2653)
    <223> Flank1 Del3

45
    <220>
    <221> promoter
    <222> (2817)..(2969)
    <223> lac promoter

50
    <220>
    <221> gene
    <222> (2818)..(3170)
    <223> LacZ

55
    <220>
    <221> promoter
    <222> (2843)..(2863)
    <223> T3

```

<220>
 <221> promoter
 <222> (2988)..(3008)
 <223> T7

5

<220>
 <221> misc_feature
 <222> (3243)..(3753)
 <223> Flank2 Del3

10

<220>
 <221> gene
 <222> (4702)..(5559)
 <223> AmpR

15

<400> 15
 ctaaattgta agcgttaata ttttgtaaatttcgcgtta aatttttgtt aaatcagctc 60
 attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
 gataggggttg agtgtgtgtc cagtttgga caagagtcca ctattaaaga acgtggactc 180
 caacgtcaaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
 ctaatcaagt tttttggggt cgagggtgccg taaagcacta aatcgggaacc ctaaaggag 300
 ccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatggtgt 360
 tattgtatac tgtctaaacg cgtagtaaa acatggcgag gaaataaatc atataaaaaa 420
 tgatttcattg attaaacat gttgtgaaaa agtcaagaac gttcacattg gcggacaatc 480
 taaaaacaat acagtgttg cagatttgcc atatattgat aatgcggtat ccgatgtatg 540
 caattcactg tataaaaaga atgtatcaag aatatccaga tttgctaatt tgataaagat 600
 agatgacgat gacaagactc ctactggtgt atataattat tttaaacctt aagatgccat 660
 tcctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga 720
 taaagcgtgt gcgtgtatag agttaaattc atataaagta gccattcttc ccatggatgt 780
 ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga 840
 tgccggcacct ctcttaagaa gtgtaaccga taataatgtt attatatcta gacaccagcg 900
 tctacatgac gagcttccga gttccaattg gttcaagttt tacataagta taaagtccga 960
 ctattgttct atattatata tgggtgttga tggatctgtg atgcatgcaa tagctgataa 1020
 tagaacttac gcaaatatta gcaaaaatat attagacaat actacaatta acgatgagtg 1080
 tagatgctgt tttttgaac cacagattag gattcttgat agagatgaga tgctcaatgg 1140
 atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg taggcgaatt 1200
 tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc tttcgggtggc 1260
 tggcacatcg gtggcgggccg cttacttggc ctgactcggc agcatagaat agcgagcaac 1320

50

55

	ggcatttctca tagagcttga ccttggttga atctttgtca tgcttcaaaa tctcaatgcg	1380
5	gtgggtccaca aagtgatagt ctggcaaacg gacatccttc tttgctttgt aagtagtttt	1440
	gaaatcacat cgataatggc cacctccttc aagcaacaga gccatgttaa catcaccctt	1500
	cagcactcca tcacgtacgt acattttctc agtggatggc tcccatttaa gagtcttctt	1560
10	ctgcataacc ggaccattgg gaggaaagtt cacaccatca aaacgaatgt catagaaaaa	1620
	acagtgcgcc ctcatgctta tgttgcttgt ggcggtgcaa atgccctggt cttcataagt	1680
15	catgcttctt tcccagtgat acccctcagg aaaagtctgc ttgaaatagt cctgaatctc	1740
	tgctgggtac ttggtgaatg ccctgttgcc gtactggaac actgttgtca agatctcgta	1800
	agcgaaaggc agagggtgcgc cttcagtgac gttcagggtct aaaatctgcg ttccctcgta	1860
20	aggatttctt tttccttctc cttcaatcac gaaattatgc ccgtttacag tgcctctcat	1920
	acacagcttg atcttcatct ctgggtttaat cacactcacc attattttata ttccaaaaaa	1980
25	aaaaaataaa atttcaattt ttgccggcaa aaattgaaat tttatttttt ttttttgaa	2040
	tataaataat ggccaagcct ttgtctcaag aagaatccac cctcattgaa agagcaacgg	2100
	ctacaatcaa cagcatcccc atctctgaag actacagcgt cgccagcgca gctctctcta	2160
30	gcgacggccg catcttcact ggtgtcaatg tatatcattt tactggggga ccttgtgcag	2220
	aactcgtggt gctgggcact gctgctgctg cggcagctgg caacctgact tgtatcgtcg	2280
35	cgatcggaaa tgagaacagg ggcatcttga gccctgcgg acggtgccga cagggtgcttc	2340
	tcgatctgca tcctgggatc aaagccatag tgaaggacag tgatggacag ccgacggcag	2400
	ttgggattcg tgaattgctg ccctctggtt atgtgtggga gggctaaact agttcaatta	2460
40	acgatgagtg tagatgctgt tattttgaac cacagattag gattcttgat agagatgaga	2520
	tgctcaatgg atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg	2580
45	taggcgaatt tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc	2640
	tttcggtggc tggcccgggc tgcaggaaga catgcatcga gatctggcgc cgtttgcgca	2700
	acgcaattaa tgtgagttag ctcaactcatt aggcaccoca ggctttacac tttatgcttc	2760
50	cggctcgtat gttgtgtgga attgtgagcg gataacaatt tcacacagga aacagctatg	2820
	accatgatta cgccaagctc gaaattaacc ctcaactaaag ggaacaaaag ctggtaccgg	2880
55	gacccccctc aagatcaacg gtatcaataa gtttgatctc gaactcctgt agcccagggg	2940
	accactcgt tctogaacga ccgcgcgcgc gatggagctc caattcgccc tatagtgagt	3000
	cgtattacaa ttcactggcc gtcgtttttac aacgtcgtga ctgggaaaac cctggcgcta	3060

	cccaacttaa	tgccttgca	gcacatcccc	ctttcgccag	ctggcgtaat	agcgaagagg	3120
	cccgacccga	tgcgccttcc	caacagttgc	gcagcctgaa	tggcgaatgg	aaacttaatt	3180
5	aacacgtggc	tagcgaattc	gatatcaago	ttatcgatac	cgtcgacctc	gagggggggc	3240
	cggaaagtt	ttataggtag	ttgatagaac	aaaatacata	attttgtaaa	aataaatcac	3300
10	tttttatact	aatatgacac	gattaccaat	acttttggtta	ctaatatcat	tagtatacgc	3360
	tacacctttt	cctcagacat	ctaaaaaat	aggtgatgat	gcaactttat	catgtaatcg	3420
	aaataataca	aatgactacg	ttgttatgag	tgcttggtat	aaggagccca	attccattat	3480
15	tcttttagct	gctaaaagcg	acgtcttgta	ttttgataat	tataccaagg	ataaaatatac	3540
	ttacgactct	ccatacgatg	atctagttac	aactatcaca	attaaatcat	tgactgctag	3600
20	agatgcgggt	acttatgtat	gtgcattctt	tatgacatcg	cctacaaatg	acactgataa	3660
	agtagattat	gaagaatact	ccacagagtt	gattgtaaat	acagatagtg	aatcgactat	3720
	agacataata	ctatctggat	ctacacattc	accggaaact	agggcttcct	cgctcactga	3780
25	ctcgctgcgc	tgggtcggtc	ggctgcggcg	agcggtatca	gctcactcaa	aggcggtaat	3840
	acggttatcc	acagaatcag	gggataacgc	aggaaagaac	atgtgagcaa	aaggccagca	3900
30	aaaggccagg	aaccgtaaaa	aggccgcgtt	gctggcggtt	ttccataggc	tccgcccccc	3960
	tgacgagcat	cacaaaaaatc	gacgctcaag	tcagaggtgg	cgaaaccoga	caggactata	4020
	aagataccag	gcggtttcccc	ctggaagctc	cctcgctgcgc	tctcctgttc	cgacctgccc	4080
35	gcttaccgga	tacctgtccg	cctttctccc	ttcggaagc	gtggcgcttt	ctcatagctc	4140
	acgctgtagg	tatctcagtt	cggtgtaggt	cgttcgctcc	aagctgggct	gtgtgcacga	4200
40	accccccggt	cagcccgacc	gctgcgcctt	atccggtaac	tatcgtcttg	agtccaaccc	4260
	ggtaagacac	gacttatcgc	cactggcagc	agccactggc	aacaggatta	gcagagcgag	4320
	gtatgtaggc	ggtgctacag	agttcttgaa	gtggtggcct	aactacggct	acactagaag	4380
45	gacagtattt	ggtatctgcg	ctctgctgaa	gccagttacc	ttcggaaaaa	gagttggtag	4440
	ctcttgatcc	ggcaaaacaaa	ccaccgctgg	tagcgggtgg	ttttttgttt	gcaagcagca	4500
50	gattacgcgc	agaaaaaaag	gatctcaaga	agatcctttg	atcttttcta	cggggctctga	4560
	cgctcagtgg	aacgaaaact	cacgttaagg	gattttggct	atgagattat	caaaaaggat	4620
	cttcacctag	atccttttaa	attaaaaatg	aagtttttaa	tcaatctaaa	gtatatatga	4680
55	gtaaacttgg	tctgacagtt	accaatgctt	aatcagtgag	gcacctatct	cagcgatctg	4740
	tctatttcgt	tcattccatag	ttgcctgact	ccccgtcgtg	tagataacta	cgatacggga	4800
	gggcttacca	tctggcccca	gtgctgcaat	gataccgcga	gacccacgct	caccggctcc	4860

```

agatttatca gcaataaacc agccagccgg aagggccgag cgcagaagtg gtcctgcaac 4920
5   tttatccgcc tccatccagt ctattaattg ttgcccggaa gctagagtaa gtagttcgcc 4980
    agttaatagt ttgocgaacg ttgttgccat tgctacaggc atcgtggtgt cacgctcgtc 5040
    gtttggtatg gcttcattca gctccggttc ccaacgatca aggcgagtta catgatcccc 5100
10  catgttgtgc aaaaaagcgg ttagctcctt cggctcctcg atcgttgtca gaagtaagtt 5160
    ggccgcagtg ttatcactca tggttatggc agcactgcat aattctctta ctgtcatgcc 5220
    atccgtaaga tgcttttctg tgactgggtga gtactcaacc aagtcattct gagaatagtg 5280
15  tatgcccga cccagttgct cttgcccggc gtcaatacgg gataataccg cgccacatag 5340
    cagaacttta aaagtgtcga tcattggaaa acgttcttcg gggcgaaaac tctcaaggat 5400
20  cttaccgctg ttgagatoca gttcgatgta acccactcgt gcacccaact gatcttcagc 5460
    atcttttact ttcaccagcg tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa 5520
    aaaggggaata agggcgacac ggaaatgttg aatactcata ctcttccttt ttcaatatta 5580
25  ttgaagcatt tatcaggggtt attgtctcat gagcggatac atatttgaat gtatttagaa 5640
    aaataaacia ataggggttc cgcgcacatt tccccgaaaa gtgccac 5687
30
    <210> 16
    <211> 6096
    <212> DNA
    <213> Artificial
35
    <220>
    <223> vector vEM12

40  <220>
    <221> misc_feature
    <222> (338)..(1263)
    <223> Flank1 Del3

45  <220>
    <221> gene
    <222> (1283)..(1962)
    <223> Azami green

50  <220>
    <221> misc_feature
    <222> (2002)..(1963)
    <223> Ps

55  <220>
    <221> misc_feature
    <222> (2009)..(2048)
    <223> Ps

```

```

<220>
<221> gene
<222> (2049)..(2447)
<223> BsdR
5
<220>
<221> promoter
<222> (2454)..(2493)
<223> Ps promoter
10
<220>
<221> misc_feature
<222> (2494)..(2534)
<223> TetO2
15
<220>
<221> gene
<222> (2535)..(3383)
<223> DNase
20
<220>
<221> gene
<222> (3389)..(3579)
<223> LacZ
25
<220>
<221> promoter
<222> (3397)..(3417)
<223> T7 promoter
30
<220>
<221> misc_feature
<222> (3652)..(4162)
<223> Flank2 Del3
35
<220>
<221> gene
<222> (5111)..(5968)
<223> AmpR
40
<400> 16
ctaaattgta agcgttaata ttttggtaaa attcgcgtta aatttttggtt aaatcagctc 60
attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
45 gataggggtg agtggtgttc cagtttgga caagagtcca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
50 ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaaggagg 300
ccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatgggtg 360
tattgtatac tgtctaaacg cgttagtaaa acatggcgag gaaataaatc atataaaaaa 420
55 tgatttcatg attaaaccat gttgtgaaaa agtcaagaac gttcacattg goggacaatc 480
taaaaacaat acagtgattg cagatttgcc atatatggat aatgcggtat ccgatgtatg 540

```

	caattcactg tataaaaaga atgtatcaag aatatccaga ttgctaatt tgataaagat	600
	agatgaogat gacaagactc ctactgggtgt atataattat tttaaaccta aagatgccat	660
5	tcctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga	720
	taaagcgtgt gcgtgtatag agttaaattc atataaagta gccattcttc ccatggatgt	780
10	ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga	840
	tgcggcacct ctottaagaa gtgtaaccga taataatggt attatatcta gacaccagcg	900
	tctacatgac gagcttcoga gttccaattg gttcaagttt tacataagta taaagtccga	960
15	ctattgttct atattatata tgggtgttga tggatctgtg atgcatgcaa tagctgataa	1020
	tagaacttac gcaaataatta gcaaaaatat attagacaat actacaatta acgatgagtg	1080
20	tagatgctgt tattttgaac cacagattag gattcttgat agagatgaga tgctcaatgg	1140
	atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg tagggcaatt	1200
	tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc ttccggtggc	1260
25	tggcacatcg gtggcgggcg cttacttggc ctgactcggc agcatagaat agcgagcaac	1320
	ggcattctca tagagcttga ccttgttga atctttgtca tgcttcaaaa tctcaatgcg	1380
30	gtggtccaca aagtgatagt ctggcaaacg gacatccttc ttgctttgt aagtagtttt	1440
	gaaatcacat cgataatggc cacctccttc aagcaacaga gccatgttaa catcacctt	1500
	cagcactcca tcacgtacgt acattttctc agtggatggc tccatttaa gagtcttctt	1560
35	ctgcataacc ggaccattgg gaggaaagt cacaccatca aaacgaatgt catagaaaaa	1620
	acagtcgccc ctcatgctta tgttgcctgt ggcggtgcaa atgccttggc cttcataagt	1680
40	catgcttctt tcccagtgat acccctcagg aaaagtctgc ttgaaatagt cctgaatata	1740
	tgctgggtac ttggtgaatg ccctgttgcc gtactggaac actgttgtca agatatacga	1800
	agcgaaaggc agagggtgcgc cttcagtga gttcaggtct aaaatctgcg ttccctcgta	1860
45	aggatttcct tttccttctc cttcaatcac gaaattatgc ccgtttacag tgccctctcat	1920
	acacagcttg atcttcatct ctggtttaat cacactcacc attatttata ttccaaaaaa	1980
50	aaaaaataaa atttcaattt ttgccggcaa aaattgaaat ttattttttt ttttttggaa	2040
	tataaataat ggccaagcct ttgtctcaag aagaatccac cctcattgaa agagcaacgg	2100
	ctacaatcaa cagcatcccc atctctgaag actacagcgt cgcagcgca gctctctcta	2160
55	gcgacggccg catcttcact ggtgtcaatg tataatcattt tactggggga ccttgtgcag	2220
	aactcgtggg gctgggcact gctgctgctg cggcagctgg caacctgact tgtatcylcg	2280
	cgatcggaat tgagaacagg ggcatcttga gccctgcgg acggtgccga caggtgcttc	2340

	togatctgca	tcctgggata	aaagccatag	tgaaggacag	tgatggacag	ccgacggcag	2400
5	ttgggattcg	tgaattgctg	ccctctgggt	atgtgtggga	gggctaaact	agtaaaaatt	2460
	gaaattttat	tttttttttt	tggaatataa	atatccctat	cagtgataga	gatctcccta	2520
	tcagtgatag	agatatgagg	ggcatgaagc	tgctgggggc	gctgctggca	ctggcggccc	2580
10	tactgcaggg	ggccgtgtcc	ctgaagatcg	cagccttcaa	catccagaca	tttggggaga	2640
	ccaagatgtc	caatgccacc	ctcgtcagct	acattgtgca	gatcctgagc	cgctatgaca	2700
15	tcgccctggt	ccaggaggtc	agagacagcc	acctgactgc	cgtggggaag	ctgctggaca	2760
	acotcaatca	ggatgcacca	gacacctatc	actacgtggt	cagtggagcca	ctgggacgga	2820
	acagctataa	ggagcgctac	ctgttcgtgt	acaggcctga	ccagggtgtc	gcgggtggaca	2880
20	gctactacta	cgatgatggc	tgcgagccct	gcgggaacga	caccttcaac	cgagagccag	2940
	ccattgtcag	gttcttctcc	cggttcacag	aggtcaggga	gtttgccatt	gttcccctgc	3000
25	atgcggccccc	gggggacgca	gtagccgaga	tcgacgctct	ctatgacgtc	tacctggatg	3060
	tccaagagaa	atggggcttg	gaggacgtca	tgttgatggg	cgacttcaat	gcgggctgca	3120
	gctatgtgag	accctcccag	tggtcatcca	tccgcctgtg	gacaagcccc	accttccagt	3180
30	ggctgatccc	cgacagcgct	gacaccacag	ctacaccac	gcactgtgcc	tatgacagga	3240
	tcgtggttgc	agggatgctg	ctccgaggcg	ccgttggttc	cgactcggct	cttcccttta	3300
35	acttcagggc	tgcttatggc	ctgagtgacc	aactggccca	agccatcagt	gaccactatc	3360
	cagtggagggt	gatgctgaag	taggagctcc	aattcgcct	atagtgagtc	gtattacaat	3420
	tcactggccg	tcgttttaca	acgtcgtgac	tgggaaaacc	ctggcgttac	ccaacttaat	3480
40	cgccttgacg	cacatccccc	tttcgccagc	tggcgtaata	gcgaagaggc	ccgcaccgat	3540
	cgccttccc	aacagttgcg	cagcctgaat	ggcgaatgga	aacttaatta	acacgtggct	3600
45	agcgaattcg	atatcaagct	tatcgatacc	gtcgacctcg	aggggggggc	cggaaagttt	3660
	tataggtagt	tgatagaaca	aaatacataa	ttttgtaaaa	ataaatcact	ttttatacta	3720
	atatgacacg	attaccaata	cttttgttac	taatatacatt	agtatacgct	acaccttttc	3780
50	ctcagacatc	taaaaaaata	ggtgatgatg	caactttatc	atgtaatcga	aataatacaa	3840
	atgactacgt	tgttatgagt	gcttggtata	aggagcccaa	ttccattatt	cttttagctg	3900
55	ctaaaagcga	cgtcttgtat	tttgataatt	ataccaagga	taaaatatct	tacgactctc	3960
	catacgatga	tctagttaca	actatcacia	ttaaatcatt	gactgctaga	gatgccggta	4020
	cttatgtatg	tgcatctctt	atgacatcgc	ctacaaatga	cactgataaa	gtagattatg	4080

	aagaatactc cacagagttg attgtaaata cagatagtga atcgactata gacataatac	4140
	tatctggatc tacacattca ccggaaaacta gggtctcctc gctcactgac tcgctgcgct	4200
5	cggtcggtcg gctgcggcga gcggtatcag ctcaactcaa ggccgtaata cggttatcca	4260
	cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa aaggccagga	4320
	accgtaaaaa ggccgcgttg ctggcgtttt tccataggct ccgccccct gacgagcatc	4380
10	acaaaaatcg acgtcgaagt cagagggtggc gaaacccgac aggactataa agataccagg	4440
	cgtttcccc tggaagctcc ctcgctgcgt ctctgttcc gacctgccg cttaccggat	4500
15	acctgtccgc ctttctccct tcgggaagcg tggcgcttcc tcatagctca cgctgtagg	4560
	atctcagttc ggtgtaggtc gtctgctcca agctgggctg tgtgcacgaa cccccgttc	4620
	agcccgaccg ctgcgcctta tccggtaact atcgctctga gtccaaccg gtaagacag	4680
20	acttatcgcc actggcagca gccactggta acaggattag cagagcgagg tatgtaggcg	4740
	gtgctacaga gttcttgaag tgggtggccta actacggcta cactagaagg acagtatttg	4800
25	gtatctgcgc tctgctgaag ccagttacct tcggaaaaag agttggtagc tcttgatccg	4860
	gcaaacaaac caccgctggt agcggtggtt tttttgttg caagcagcag attacgcgca	4920
	gaaaaaaagg atctcaagaa gatcctttga tcttttctac ggggtctgac gctcagtgg	4980
30	acgaaaactc acgttaaggg attttggtca tgagattatc aaaaaggatc ttcacctaga	5040
	tccttttaaa ttaaaaatga agttttaaat caatctaaag tatatatgag taaacttggt	5100
35	ctgacagtta ccaatgctta atcagtgagg cacctatctc agcgatctgt ctatttcgtt	5160
	catccatagt tgctgactc ccgctcgtgt agataactac gatacgggag ggcttaccat	5220
	ctggccccag tgctgcaatg ataccgcgag acccacgctc accggctcca gatttatcag	5280
40	caataaacca gccagccgga agggccgagc gcagaagtgg tcctgcaact ttatccgcct	5340
	ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca gttaatagtt	5400
45	tgcgcaacgt tgttgccatt gctacaggca tcgtggtgct acgctcgtcg tttggtatgg	5460
	cttcattcag ctccggttcc caacgatcaa ggccagttac atgatcccc atgttgtgca	5520
	aaaaagcggg tagctccttc ggtcctccga tcgttgctcag aagtaagttg gccgcagtgt	5580
50	tatcactcat ggttatggca gcaactgcata attctcttac tgtcatgcca tccgtaagat	5640
	gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt atgcggcgac	5700
55	cgagttgctc ttgcccgcg tcaatacggg ataataccgc gccacatagc agaactttaa	5760
	aagtgtcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc ttaccgctgt	5820
	tgagatccag ttgatgtaa ccactcgtg caccctactg atcttcagca tcttttaact	5880

```

tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa aaggggaataa 5940
gggcgacacg gaaatgttga atactcatac tcttcctttt tcaatattat tgaagcattt 6000
5 atcagggtta ttgtctcatg agcggataca tatttgaatg tatttagaaa aataaacaaa 6060
taggggttcc ggcgcacattt ccccgaaaag tgccac 6096

10 <210> 17
    <211> 6585
    <212> DNA
    <213> artificial
15 <220>
    <223> vector vEM31

20 <220>
    <221> misc_feature
    <222> (338)..(1263)
    <223> Flank1 Del3

25 <220>
    <221> gene
    <222> (1283)..(1962)
    <223> Azami green

30 <220>
    <221> misc_feature
    <222> (2002)..(1963)
    <223> Ps

35 <220>
    <221> misc_feature
    <222> (2009)..(2048)
    <223> Ps

40 <220>
    <221> gene
    <222> (2049)..(2447)
    <223> BdsR

45 <220>
    <221> promoter
    <222> (2454)..(2493)
    <223> Ps promoter

50 <220>
    <221> misc_feature
    <222> (2494)..(2524)
    <223> TetO2

55 <220>
    <221> gene
    <222> (2535)..(3383)
    <223> DNase

```

```

<220>
<221> promoter
<222> (3390)..(3429)
<223> Ps promoter
5
<220>
<221> gene
<222> (3430)..(4086)
<223> TetR
10
<220>
<221> misc_feature
<222> (4141)..(4651)
<223> Flank2 Del3
15
<220>
<221> gene
<222> (5600)..(6457)
<223> AmpR
20
<400> 17
ctaaattgta agcgttaata ttttggtaaa attcgcgtta aatttttgtt aaatcagctc 60
atattttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
25 gatagggttg agtggtgttc cagtttgga caagagtcca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
30 ctaatcaagt tttttggggt cgagggtgcc taaagcacta aatcggaacc ctaaaggag 300
cccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatggtgt 360
tattgtatac tgtctaaacg cgtagtaaaa acatggcgag gaaataaatc atataaaaaa 420
35 tgatttcatt attaaaccat gttgtgaaaa agtcaagaac gttcacattg gcggacaatc 480
taaaaacaat acagtgattg cagatttgcc atatatggat aatgcggtat ccgatgtatg 540
40 caattcactg tataaaaaaga atgtatcaag aatatccaga ttgctaatt tgataaagat 600
agatgacgat gacaagactc ctactggtgt atataattat tttaaacctt aagatgccat 660
tcctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga 720
45 taaagcgtgt gcgtgtatag agttaaattc atataaagta gccattcttc ccatggatgt 780
ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga 840
50 tgcggcacct ctcttaagaa gtgtaaccga taataatggt attatatcta gacaccagcg 900
tctacatgac gagcttccga gttccaattg gttcaagttt tacataagta taaagtccga 960
ctattgttct atattatata tgggtgttga tggatctgtg atgcatgcaa tagctgataa 1020
55 tagaacttac gcaaatatta gcaaaaatat attagacaat actacaatta acgatgagtg 1080
tagatgctgt tattttgaac cacagattag gattcttgat agagatgaga tgctcaatgg 1140

```

	atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg taggcgaatt	1200
	tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc tttcgggtggc	1260
5	tggcacatcg gtggcggccg cttacttggc ctgactcggc agcatagaat agcgagcaac	1320
	ggcattctca tagagcttga ccttgttgta atctttgtca tgcttcaaaa tctcaatgcg	1380
	gtggtccaca aagtgatagt ctggcaaacg gacatccttc tttgctttgt aagtagtttt	1440
10	gaaatcacat cgataatggc cacctccttc aagcaacaga gccatgttaa catcaccctt	1500
	cagcactcca tcacgtacgt acatcttctc agtggatggc tcccatctaa gagtcttctt	1560
15	ctgcataacc ggaccattgg gaggaaagt caccacatca aaacgaatgt catagaaaaa	1620
	acagtcgccc ctcatgctta tgttgcttgt ggcggtgcaa atgccctggt cttcataagt	1680
	catgcttctt toccagtgat accctcagg aaaagtctgc ttgaaatagt cctgaatctc	1740
20	tgctgggtac ttggtgaatg ccctgttgcc gtactggaac actgttgctca agatatcgta	1800
	agcgaaaggc agaggtgcgc cttcagtgc gttcaggtct aaaatctgcg ttcctctgta	1860
25	aggatttctt tttccttctc cttcaatcac gaaattatgc ccgtttacag tgcctctcat	1920
	acacagcttg atcttcatct ctggtttaat cacactcacc attatttata ttccaaaaaa	1980
	aaaaaataaa atttcaattt ttgccggcaa aaattgaaat tttatttttt ttttttgtaa	2040
30	tataaataat ggccaagcct ttgtctcaag aagaatccac cctcattgaa agagcaacgg	2100
	ctacaatcaa cagcatcccc atctctgaag actacagcgt cgccagcgca gctctctcta	2160
35	gcgacggccg catcttcaact ggtgtcaatg tatatcattt tactggggga cctgtgtcag	2220
	aactcgtggt gctgggcact gctgctgctg cggcagctgg caacctgact tgtatcgtcg	2280
	cgatcggaaa tgagaacagg ggcattctga gccctcggg acggtgccga caggtgcttc	2340
40	tcgatctgca tcctgggatc aaagccatag tgaaggacag tgatggacag ccgacggcag	2400
	ttgggattcg tgaattgctg ccctctggtt atgtgtggga gggctaaact agtaaaaatt	2460
45	gaaattttat tttttttttt tggaatataa atatccctat cagtgataga gatctcccta	2520
	tcagtgatag agatatgagg ggcattgaag tgctgggggc gctgctggca ctggcggccc	2580
	tactgcaggg ggccgtgtcc ctgaagatcg cagccttcaa catccagaca tttggggaga	2640
50	ccaagatgtc caatgccacc ctgcgcagct acattgtgca gatcctgagc cgctatgaca	2700
	tcgccttggg ccaggaggtc agagacagcc acctgactgc cgtggggaag ctgctggaca	2760
55	acctcaatca ggatgcacca gacacctatc actacgtggt cagtgagcca ctgggacgga	2820
	acagctataa ggagcgctac ctgttcgtgt acaggcctga ccagggtgtct gcggtggaca	2880
	gctactacta cgatgatggc tgcgagccct gcgggaacga caccttcaac cgagagccag	2940

	ccattgtcag gttcttctcc cggttcacag aggtcaggga gtttgccatt gttcccctgc	3000
5	atgcggcccc gggggacgca gtagccgaga tgcacgctct ctatgacgtc tacctggatg	3060
	tccaagagaa atggggcttg gaggaagtca tgttgatggg cgacttcaat gcgggctgca	3120
	gctatgtgag accctcccag tggtcattca tccgcctgtg gacaagcccc accttccagt	3180
10	ggctgatccc cgacagcgct gacaccacag ctacacccac gcactgtgcc tatgacagga	3240
	tcgtggttgc agggatgctg ctccgaggcg ccgttgttcc cgactcggct ctcccttta	3300
	acttccaggc tgccatggc ctgagtgacc aactggccca agccatcagt gaccactatc	3360
15	cagtggaggt gatgctgaag taggagctca aaaattgaaa ttttattttt tttttttgga	3420
	atataataaa tgtctagatt agataaaaagt aaagtgatta acagcgcatt agagctgctt	3480
20	aatgaggtcg gaatcgaagg tttaacaacc cgtaaaactcg cccagaagct aggtgtagag	3540
	cagcctacat tgtattggca tgtaaaaaat aagcgggctt tgctcgacgc cttagccatt	3600
	gagatgttag ataggcacca tactcacttt tgccctttag aaggggaaag ctggcaagat	3660
25	tttttacgta ataacgctaa aagttttaga tgtgctttac taagtcatcg cgatggagca	3720
	aaagtacatt taggtacacg gcctacagaa aaacagtatg aaactctcga aaatcaatta	3780
30	gcctttttat gccaaacaagg tttttcacta gagaatgcat tatatgcact cagcgtgtg	3840
	gggcatttta ctttaggttg cgtattggaa gatcaagagc atcaagtgcg taaagaagaa	3900
	agggaaacac ctactactga tagtatgccg ccattattac gacaagctat cgaattattt	3960
35	gatcaccaag gtgcagagcc agccttctta ttcggccttg aattgatcat atgcggatta	4020
	gaaaaacaac ttaaattgtga aagtgggtcc gcgtacagcg gatcccggga attcagatct	4080
40	tattaagcta gcgaattcga tatcaagctt atcgataccg tcgacctcga gggggggccc	4140
	ggaaagtttt ataggtagtt gatagaacaa aatacataat tttgtaaaaa taaatcactt	4200
	tttatactaa tatgacacga ttaccaatac ttttgttact aatatcatta gtatacgcta	4260
45	caccttttcc tcagacatct aaaaaaatag gtgatgatgc aactttatca tgtaatcgaa	4320
	ataatacaaa tgactacgtt gttatgagtg cttggtataa ggagcccaat tccattattc	4380
50	ttttagctgc taaaagcgac gtcttgtatt ttgataatta taccaaggat aaaatatctt	4440
	acgactctcc atacgatgat ctagttacaa ctatcacaat taaatcattg actgctagag	4500
	atgccggtac ttatgtatgt gcattcttta tgacatcgcc taaaaatgac actgataaag	4560
55	tagattatga agaatactcc acagagttga ttgtaaatac agatagtga tgcactatag	4620
	acataatact atctggatct acacattcac cggaaactag ggcttctctg ctactgact	4680

	cgctgcgctc	ggtcgttcgg	ctgcggcgag	cggatcagc	tcactcaaag	gcggtaatac	4740
	ggttatccac	agaatcaggg	gataacgcag	gaaagaacat	gtgagcaaaa	ggccagcaaa	4800
5	agggccaggaa	ccgtaaaaag	gccgcgttgc	tggcgttttt	ccataggctc	cgccccctg	4860
	acgagcatca	caaaaatcga	cgctcaagtc	agaggtggcg	aaacccgaca	ggactataaa	4920
10	gataccaggc	gtttcccoct	ggaagctccc	tcgtgcgctc	tcctgttcog	accctgccgc	4980
	ttaccggata	cctgtccgcc	tttctccctt	cgggaagcgt	ggcgctttct	catagctcac	5040
	gctgtaggta	tctcagttcg	gtgtaggtcg	ttcgctccaa	gctgggctgt	gtgcacgaac	5100
15	cccccgttca	gcccgcgcgc	tgcgccttat	cgggtaacta	tcgtcttgag	tccaaccggg	5160
	taagacacga	cttatcgcca	ctggcagcag	ccactggtaa	caggattagc	agagcgaggt	5220
20	atgtaggcgg	tgctacagag	ttcttgaagt	ggtggcctaa	ctacggctac	actagaagga	5280
	cagtatttgg	tatctgcgct	ctgctgaagc	cagttacctt	cggaaaaaga	gttggtagct	5340
	cttgatccgg	caaacaaacc	accgctggta	gcgggtggtt	ttttgtttgc	aagcagcaga	5400
25	ttacgcgcag	aaaaaaagga	tctcaagaag	atcctttgat	cttttctacg	gggtctgacg	5460
	ctcagtggaa	cgaaaaactca	cgtaagggga	ttttggtcat	gagattatca	aaaaggatct	5520
30	tcacctagat	cctttttaaat	taaaaatgaa	gttttaaatc	aatctaaagt	atatatgagt	5580
	aaacttggtc	tgacagttac	caatgcttaa	tcagtgaggc	acctatctca	gcgatctgtc	5640
	tatttcggtc	atccatagtt	gcctgactcc	ccgtcgtgta	gataactacg	atacgggagg	5700
35	gcttaccatc	tggccccagt	gctgcaatga	taccgcgaga	cccacgctca	ccggctccag	5760
	atztatcagc	aataaaccag	ccagccggaa	gggccgagcg	cagaagtggg	cctgcaactt	5820
40	tatccgcctc	catccagtct	attaattggt	gccgggaagc	tagagtaagt	agttcgccag	5880
	ttaatagttt	gcgcaacggt	gttgccattg	ctacaggcat	cgtgggtgtca	cgctcgctgt	5940
	ttggatggc	ttcattcagc	tccggttccc	aacgatcaag	gcgagttaca	tgatccccca	6000
45	tgttgtgcaa	aaaagcgggt	agctccttcg	gtcctccgat	cgttgtcaga	agtaagttgg	6060
	ccgcagtgtt	atcactcatg	gttatggcag	cactgcataa	ttctcttact	gtcatgccat	6120
50	ccgtaagatg	cttttctgtg	actgggtgag	actcaaccaa	gtcattctga	gaatagtgtg	6180
	tgccggcgacc	gagttgctct	tgcccggcgt	caatacggga	taataccgcg	ccacatagca	6240
	gaactttaaa	agtgtctatc	attggaaaac	gttcttcggg	gcgaaaactc	tcaaggatct	6300
55	taccgctgtt	gagatccagt	tcgatgtaac	ccactcgtgc	acccaactga	tcttcagcat	6360
	cttttacttt	caccagcgtt	tctgggtgag	caaaaacagg	aaggcaaat	gcccgaaaaa	6420
	agggataaag	ggcgacacgg	aatgttgaa	tactcatact	cttccttttt	caatattatt	6480

```

gaagcatttta tcaggggttat tgtctcatga gcggtatacat atttgaatgt atttagaaaa 6540
ataaacaaaat aggggggttcg cgcacatttc cccgaaaagt gccac 6585
5
<210> 18
<211> 3014
<212> DNA
10 <213> artificial

<220>
<223> plasmid

15
<220>
<221> promoter
<222> (622)..(642)
<223> T7 promoter

20
<220>
<221> misc_feature
<222> (680)..(742)
<223> p7.5

25
<220>
<221> promoter
<222> (824)..(844)
<223> T3 promoter

30
<220>
<221> promoter
<222> (870)..(991)
<223> lac promoter

35
<220>
<221> gene
<222> (2029)..(2886)
<223> AmpR

40
<400> 18
ctaaattgta agcgttaata ttttgtaaa attcgcgtta aatttttgtt aaatcagctc 60
attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
45 gataggggttg agtgttgttc cagtttgga caagagtcca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
50 ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaaggag 300
cccccgattt agagcttgac ggggaaagcc ggcgaaacgtg gcgagaaagg aagggaagaa 360
agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac 420
55 cacacccgcc gcgcttaatg cgccgctaca gggcgcgctcc cattcgccat tcaggctgcg 480
caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg 540

```

	gggatgtgct gcaagggcat taagttgggt aacgccaggg ttttcccagt cacgacgttg	600
	taaaacgacg gccagtgaat tgtaatacga ctactatag ggcgaattgg agctccaccg	660
5	cggtggcggc cgctctagaa taaataataa atacaataat taatttctcg taaaagtaga	720
	aaatatattc taattttattg caggatcccc cgggctgcag gaattcgata tcaagcttat	780
10	cgataccgtc gacctcgagg gggggcccg ggtaccagcttt tgttcccttt agtgagggtt	840
	aatttcgagc ttggcgtaat catggtcata gctgtttcct gtgtgaaatt gttatccgct	900
	cacaattcca cacaacatac gagccggaag cataaagtgt aaagcctggg gtgcctaata	960
15	agtgagctaa ctacattaa ttgcgttgcg ctactgccc gctttccagt cgggaaacct	1020
	gtcgtgccag ctgcattaat gaatcgcca acgcgcgggg agaggcggtt tgcgtattgg	1080
20	gcgtctttcc gcttctcgc tcaactgactc gctgcgctcg gtcgttcggc tgcggcgagc	1140
	ggtatcagct cactcaaagg cggtaatcag gttatccaca gaatcagggg ataacgcagg	1200
	aaagaacatg tgagcaaaag gccagcaaaa ggccaggaac cgtaaaaagg ccgcgttgct	1260
25	ggcggttttt cataggtctc gccccctga cgagcatcac aaaaatcgac gctcaagtca	1320
	gaggtggcga aacccgacag gactataaag ataccaggcg tttccccctg gaagctccct	1380
30	cgtgcgctct cctgttcoga ccctgccgt taccggatac ctgtccgctt ttctcccttc	1440
	gggaagcggt gcgctttctc atagctcacg ctgtaggtat ctgagttcgg tgtaggtcgt	1500
	tcgctccaag ctgggctgtg tgcacgaacc ccccgctcag cccgaccgct gcgccttata	1560
35	cgtaactat cgtcttgagt ccaaccgggt aagacacgac ttatcgccac tggcagcagc	1620
	cactggtaac aggattagca gagcgaggta tgtaggcggg gctacagagt tcttgaagtg	1680
40	gtggcctaac tacggctaca ctagaaggac agtatttggg atctgcgctc tgctgaagcc	1740
	agttaccttc ggaaaaagag ttggtagctc ttgatccggc aaacaaacca ccgctggtag	1800
	cggtggtttt tttgtttgca agcagcagat tacgcgcaga aaaaaaggat ctcaagaaga	1860
45	tcctttgatc ttttctacgg ggtctgacgc tcagtggaac gaaaactcac gttaagggat	1920
	tttggtcata agattatcaa aaaggatctt cacctagatc cttttaaat aaaaatgaag	1980
50	ttttaaatca atctaaagta tatatgagta aacttgggtc gacagttacc aatgcttaat	2040
	cagtgaggca cctatctcag cgatctgtct atttcgttca tccatagttg cctgactccc	2100
	cgtcgtgtag ataactacga tacgggaggg cttaccatct ggccccagtg ctgcaatgat	2160
55	accgcgagac ccacgctcac cggctccaga ttatcagca ataaaccagc cagccggaag	2220
	ggccgagcgc agaagtggtc ctgcaacttt atccgcctcc atccagtcta Llaattgttg	2280
	ccgggaagct agagtaagta gttcgccagt taatagtttg cgcaacgttg ttgccattgc	2340

	tacaggcatc gtgggtgtcac gctcgtcggt tggtatggct tcattcagct ccgggttecca	2400
	acgatcaagg cgagttacat gatcccccatt gttgtgcaaa aaagcgggta gtccttcggg	2460
5	tcctccgata gttgtcagaa gtaagttggc cgcagtgtta tcactcatgg ttatggcagc	2520
	actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctgggtgagta	2580
10	ctcaaccaag tcattctgag aatagtgtat ggggogacgg agttgctctt gcccggcgtc	2640
	aatacgggat aataccgcgc cacatagcag aactttaaaa gtgctcatca ttggaaaacg	2700
	ttcttcgggg cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc	2760
15	cactcgtgca cccaactgat cttcagcatc ttttactttc accagcgttt ctgggtgagc	2820
	aaaaacagga aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga aatgttgaat	2880
20	actcatactc ttcctttttc aatattattg aagcatttat cagggttatt gtctcatgag	2940
	cggatacata tttgaatgta tttagaaaaa taaacaaata ggggttccgc gcacatttcc	3000
	ccgaaaagtg ccac	3014
25		
	<210> 19	
	<211> 3024	
	<212> DNA	
30	<213> artificial	
	<220>	
	<223> plasmid	
35		
	<220>	
	<221> promoter	
	<222> (625)..(645)	
	<223> T7 promoter	
40		
	<220>	
	<221> promoter	
	<222> (683)..(752)	
	<223> H5 promoter	
45		
	<220>	
	<221> promoter	
	<222> (834)..(854)	
	<223> T3 promoter	
50		
	<220>	
	<221> promoter	
	<222> (880)..(1001)	
	<223> lac promoter	
55		
	<220>	
	<221> gene	
	<222> (2039)..(2896)	
	<223> AmpR	

<400> 19
 ctaaattgta agcggttaata ttttggttaaa attcgcgtta aatttttgtt aaatcagctc 60
 5 attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
 gataggggttg agtggttggtc cagtttgga caagagtcca ctattaaaga acgtggactc 180
 caacgtcaaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
 10 ctaatcaagt tttttggggg cgaggtgccg taaagcacta aatcggaacc ctaaagggag 300
 ccccgattt agagcttgac ggggaaagcc ggcgaaacgtg gcgagaaagg aagggagaa 360
 15 agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac 420
 cacacccgcc gcgcttaatg cgccgctaca gggcgcgctc cattcgccat tcaggctgcg 480
 caactgttgga gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg 540
 20 gggatgtgct gcaaggcgat taagtgggt aacgccaggg tttcccagt cagcagcttg 600
 taaaacgacg gccagtgagc gcgcgtaata cgactcacta tagggcgaat tggagctcca 660
 25 ccgcggtggc ggccgctcta gaaaaaaatg aaaataaata caaaggttct tgagggttgt 720
 gttaaattga aagcgagaaa taatcataaa ttggatcccc cgggctgcag gaattcgata 780
 tcaagcttat cgataccgct gacctcgagg gggggcccgg taccagcttt tgttcccttt 840
 30 agtgagggtt aatttcgagc ttggcgtaat catggtcata gctgtttcct gtgtgaaatt 900
 gttatccgct cacaattcca cacaacatac gagccggaag cataaagtgt aaagcctggg 960
 35 gtgcctaattg agtgagctaa ctcacattaa ttgcgttgcg ctactgccc gctttccagt 1020
 cgggaaacct gtcgtgccag ctgcattaat gaatcggcca acgcgcgggg agaggcggtt 1080
 tgcgtattgg gcgctcttcc gcttctcgc tcaactgactc gctgcgctcg gtcgttcggc 1140
 40 tgcggcgagc ggtatcagct cactcaaagg cggtaatagc gttatccaca gaatcagggg 1200
 ataacgcagg aaagaacatg tgagcaaaag gccagcaaaa ggccaggaac cgtaaaaagg 1260
 45 ccgcgttgct ggcgtttttc cataggctcc gccccctga cgagcatcac aaaaatcgac 1320
 gctcaagtca gaggtggcga aacccgacag gactataaag ataccaggcg tttccccctg 1380
 gaagctccct cgtgcgctct cctgttccga ccctgccgct taccggatac ctgtccgct 1440
 50 ttctcccttc gggaagcgtg gcgctttctc atagctcacg ctgtaggtat ctgagttcgg 1500
 tgtaggtcgt tcgctccaag ctgggctgtg tgcacgaacc cccggttcag cccgaccgct 1560
 55 gcgccttatc cggttaactat cgtcttgagt ccaaccgggt aagacacgac ttatcgccac 1620
 tggcagcagc cactggtaac aggattagca gagcgaggtg ttagggcggt gctacagagt 1680
 tcttgaagtg gtggcctaac tacggctaca ctagaaggac agtatttggt atctgcgctc 1740

```

tgctgaagcc agttaccttc ggaaaaagag ttggtagctc ttgatccggc aaacaaacca 1800
5  ccgctggttag cggtggtttt tttgtttgca agcagcagat tacgcgcaga aaaaaaggat 1860
   ctcaagaaga tcttttgatc ttttctacgg ggtctgacgc tcagtggaac gaaaactcac 1920
   gttaagggat tttggtcatg agattatcaa aaaggatcctt cacctagatc ctttttaaatt 1980
10  aaaaatgaag ttttaaatac atctaaagta tatatgagta aacttgggtc gacagttacc 2040
   aatgcttaat cagtgaaggc cctatctcag cgatctgtct atttcgttca tccatagttg 2100
   cctgactccc cgtcgtgtag ataactacga tacgggaggg cttaccatct ggccccagtg 2160
15  ctgcaatgat accgcgagac ccacgctcac cggctccaga tttatcagca ataaaccagc 2220
   cagccggaag ggccgagcgc agaagtggtc ctgcaacttt atccgcctcc atccagtcta 2280
20  ttaattgttg ccgggaagct agagtaagta gttcgccagt taatagtttg cgcaacgttg 2340
   ttgccattgc tacaggcatc gtgggtgtcac gctcgtcgtt tggatatggc tcattcagct 2400
   ccggttccca acgatcaagg cgagttacat gatcccccat gttgtgcaaa aaagcggtta 2460
25  gctccttcgg tcttccgacg gttgtcagaa gtaagttggc cgcagtgtta tcaactcatgg 2520
   ttatggcagc actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga 2580
30  ctggtgagta ctcaaccaag tcattctgag aatagtgtat gcggcgaccg agttgctctt 2640
   gccggcgctc aatacgggat aataccgcgc cacatagcag aactttaaaa gtgctcatca 2700
   ttggaaaacg ttcttcgggg cgaaaactct caaggatcct accgctgttg agatccagtt 2760
35  cgatgtaacc cactcgtgca cccaactgat cttcagcatc ttttactttc accagcgttt 2820
   ctgggtgagc aaaaacagga aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga 2880
40  aatgttgaat actcatactc ttcctttttc aatattattg aagcatttat caggggttatt 2940
   gtctcatgag cggatacata tttgaatgta tttagaaaaa taaacaaata ggggttccgc 3000
   gcacatttcc ccgaaaagtg ccac 3024
45

```

<210> 20

<211> 3667

<212> DNA

50 <213> artificial

<220>

<223> vector pEM12 (7.5)

55

<220>

<221> promoter

<222> (622)..(642)

<223> T7 promoter

```

<220>
<221> misc_feature
<222> (680)..(742)
5 <223> p7.5

<220>
<221> gene
<222> (799)..(1455)
10 <223> TetR

<220>
<221> promoter
<222> (1477)..(1497)
15 <223> T3 promoter

<220>
<221> promoter
<222> (1523)..(1644)
20 <223> lac promoter

<220>
<221> gene
<222> (2682)..(3539)
25 <223> AmpR

<400> 20
ctaaattgta agcggttaata ttttggttaa attcgcgtta aatttttgtt aaatcagctc 60
30 atttttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
gataggggttg agtggtgttc cagtttgga caagagtoca ctattaaaga acgtggactc 180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
35 ctaatcaagt tttttggggg cgaggtgccg taaagcacta aatcggaacc ctaaagggag 300
cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aaggggaagaa 360
40 agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac 420
cacaccgcc gcgcttaatg cgccgctaca gggcgcgctc cattcgccat tcaggctgcg 480
caactgttgg gaagggcgat cgggtgcggc ctcttcgcta ttacgccagc tggcgaaagg 540
45 gggatgtgct gcaaggcgat taagttgggt aacgccaggg ttttccagc cagcagcttg 600
taaaacgacg gccagtgaat tgtaatacga ctactatag ggcgaattgg agctccaccg 660
50 cgggtggcggc cgctctagaa taaataataa atacaataat taattttctcg taaaagtaga 720
aaatatattc taattttattg caggatcccc cgggctgcag gaattcgata tcaagcttat 780
cgataccgtc gaccgcgagat gtctagatta gataaaagta aagtgattaa cagcgcatta 840
55 gagctgctta atgaggtcgg aatcgaaggt ttaacaaccc gtaaactcgc ccagaagcta 900
gggtgtagagc agcctacatt gtattggcat gtaaaaaata agcgggcttt gctcgacgcc 960

```

	ttagccattg	agatggtaga	taggcacat	actcactttt	gccctttaga	aggggaaagc	1020
	tggcaagatt	ttttacgtaa	taacgctaaa	agtttttagat	gtgctttact	aagtcacgc	1080
5	gatggagcaa	aagtacattt	aggtacacgg	cctacagaaa	aacagtatga	aactctcgaa	1140
	aatcaattag	cctttttatg	ccaacaaggt	ttttcactag	agaatgcatt	atatgcactc	1200
	agcgctgtgg	ggcattttac	tttaggttgc	gtattggaag	atcaagagca	tcaagtcgct	1260
10	aaagaagaaa	gggaaacacc	tactactgat	agtatgccgc	cattattacg	acaagctatc	1320
	gaattatttg	atcaccaagg	tgcagagcca	gccttcttat	tcggccttga	attgatcata	1380
15	tgcggattag	aaaaacaact	taaatgtgaa	agtgggtccg	cgtacagcgg	atcccgggaa	1440
	ttcagatctt	attaagggcc	cggtaccagc	ttttgttccc	tttagtgagg	gttaatttcg	1500
	agcttggcgt	aatcatggtc	atagctgttt	cctgtgtgaa	attgttatcc	gctcacaatt	1560
20	ccacacaaca	tacgagccgg	aagcataaag	tgtaaagcct	ggggtgccta	atgagtgagc	1620
	taactcacat	taattgcgtt	gcgctcactg	cccgccttcc	agtcgggaaa	cctgtcgtgc	1680
25	cagctgcatt	aatgaatcgg	ccaacgcgcg	gggagaggcg	gtttgcgtat	tgggcgctct	1740
	tccgcttcc	cgctcactga	ctcgctgcgc	tcggtcgttc	ggctgcggcg	agcggtatca	1800
	gctcactcaa	aggcggtaat	acggttatcc	acagaatcag	gggataacgc	aggaaagaac	1860
30	atgtgagcaa	aaggccagca	aaaggccagg	aaccgtaaaa	aggccgcgtt	gctggcgttt	1920
	ttccataggg	tccgcccccc	tgacgagcat	cacaaaaatc	gacgctcaag	tcagaggtgg	1980
35	cgaaaccocga	caggactata	aagataccag	gcgtttcccc	ctggaagctc	cctcgtgcgc	2040
	tctcctgttc	cgaccctgcc	gcttaccgga	tacctgtccg	cctttctccc	ttcgggaagc	2100
	gtggcgcttt	ctcatagctc	acgctgtagg	tatctcagtt	cgggtgtagg	cgttcgctcc	2160
40	aagctgggct	gtgtgcacga	acccccggt	cagcccgacc	gctgcgcctt	atccggtaac	2220
	tatcgtcttg	agtccaaccc	ggtaagacac	gacttatcgc	cactggcagc	agccactgg	2280
45	aacaggatta	gcagagcgag	gtatgtaggc	ggtgctacag	agttcttgaa	gtgggtggc	2340
	aactacggct	acactagaag	gacagtattt	ggtatctgcg	ctctgctgaa	gccagttacc	2400
	ttcggaaaaa	gagttggtag	ctcttgatcc	ggcaaacaaa	ccaccgctgg	tagcgggtgg	2460
50	ttttttgttt	gcaagcagca	gattacgcgc	agaaaaaag	gatctcaaga	agatcctttg	2520
	atcttttcta	cggggtctga	cgctcagtgg	aacgaaaact	cacgttaagg	gattttggtc	2580
55	atgagattat	caaaaaggat	cttcacctag	atccttttaa	attaaaaatg	aagttttaaa	2640
	tcaatctaaa	gtatatatga	gtaaacttgg	tctgacagtt	accaatgctt	aatcagtgag	2700
	gcacctatct	cagcgatctg	tctatttcgt	tcattccatag	ttgcctgact	ccccgctcgtg	2760

```

tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat gataccgcga 2820
gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg aagggccgag 2880
5  cgcagaagtg gtcttgcaac tttatccgcc tccatccagt ctattaattg ttgccgggaa 2940
gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat tgctacaggc 3000
10 atcgtgggtg cacgctcgtc gtttggtatg gcttcattca gctccggttc ccaacgatca 3060
aggcgagtta catgatcccc catgttggtc aaaaaagcgg ttagctcctt cggctctccg 3120
atcgttggtc gaagtaagtt ggcgcgagt ttatcactca tggttatggc agcactgcat 3180
15 aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga gtactcaacc 3240
aagtcattct gagaatagtg tatgcggcga ccgagttgct cttgcccggc gtcaatacgg 3300
20 gataataccg cgccacatag cagaacttta aaagtgtc tcatggaaa acgttctctg 3360
ggcgaaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta acccactcgt 3420
gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg agcaaaaaaca 3480
25 ggaaggcaaa atgccgcaaa aaagggaata agggcgacac ggaaatgttg aatactcata 3540
ctcttctttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat gagcggatac 3600
30 atatttgaat gtatttagaa aaataaacia ataggggttc cgcgcacatt tccccgaaaa 3660
gtgccac 3667

35 <210> 21
    <211> 3695
    <212> DNA
    <213> artificial

40 <220>
    <223> vector pEM13 (H5)

    <220>
45 <221> promoter
    <222> (625)..(645)
    <223> T7 promoter

    <220>
50 <221> promoter
    <222> (683)..(752)
    <223> H5 promoter

    <220>
55 <221> gene
    <222> (786)..(1442)
    <223> TetR

    <220>

```

<221> promoter
 <222> (1505)..(1525)
 <223> T3 promoter

5 <220>
 <221> promoter
 <222> (1551)..(1672)
 <223> lac promoter

10 <220>
 <221> gene
 <222> (2710)..(3567)
 <223> AmpR

15 <400> 21
 ctaaattgta agcgttaata ttttgtaa attcgcgtta aatttttgtt aaatcagctc 60
 attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
 20 gataggggtt agtggtgttc cagtttgga caagagtcca ctattaaaga acgtggactc 180
 caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
 ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcgggaacc cttaaaggag 300
 25 ccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa 360
 agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac 420
 30 cacaccgcgc gcgcttaatg cgccgctaca gggcgcgctc cattcgccat tcaggctgcg 480
 caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg 540
 gggatgtgct gcaaggcgat taagttgggt aacgccaggg ttttccagc cagcagcttg 600
 35 taaaacgacg gccagtgagc gcgcgtaata cgactcacta tagggcgaat tggagctcca 660
 ccgcgggtggc ggccgctcta gaaaaaaatg aaaataaata caaaggttct tgagggttgt 720
 40 gttaaattga aagcgagaaa taatcataaa ttggatcccc cgggctgcag gaattcgatc 780
 tcgagatgtc tagattagat aaaagtaaag tgattaacag cgcattagag ctgcttaatg 840
 aggtcggaat cgaaggttta acaaccgta aactcgccca gaagctaggt gtagagcagc 900
 45 ctacattgta ttggcatgta aaaaataagc gggctttgct cgacgcctta gccattgaga 960
 tgttagatag gcaccatact cacttttgcc ctttagaagg ggaaagctgg caagattttt 1020
 50 tacgtaataa cgctaaaagt tttagatgtg ctttactaag tcacgcgat ggagcaaaag 1080
 tacatttagg tacacggcct acagaaaaac agtatgaaac tctcgaaaat caattagcct 1140
 ttttatgcca acaaggtttt tcactagaga atgcattata tgcactcagc gctgtggggc 1200
 55 attttacttt aggttgcgta ttggaagatc aagagcatca agtcgctaaa gaagaaaggg 1260
 aaacacctac tactgatagt atgccgccat tattacgaca agctatcgaa ttatttgatc 1320

	accaaggtgc agagccagcc ttcttattcg gccttgaatt gatcatatgc ggattagaaa	1380
	aacaacttaa atgtgaaagt gggtcgcgt acagcggatc cggggaattc agatcttatt	1440
5	aagggcccg atcaagctta tcgataccgt cgacctcgag ggggggcccgt gtaccagctt	1500
	ttgttccctt tagtgagggt taatttcgag cttggcgtaa tcatgggtcat agctgtttcc	1560
	tgtgtgaaat tggtatccgc tcacaattcc acacaacata cgagccggaa gcataaagt	1620
10	taaagcctgg ggtgcctaata gagtgagcta actcacatta attgcgttgc gctcactgcc	1680
	cgctttccag tcgggaaacc tgcgtgccca gctgcattaa tgaatcggcc aacgcgcggg	1740
15	gagaggcggt ttgcgtattg ggcgctcttc cgcttccctg ctcactgact cgctgcgctc	1800
	ggctgttcgg ctgcggcgag cggatcagc tcaactcaaag gcggaatac ggttatccac	1860
	agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa	1920
20	ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgccccctg acgagcatca	1980
	caaaaatcga cgctcaagtc agaggtggcg aaaccgcaca ggactataaa gataccaggc	2040
25	gtttccccct ggaagctccc tcgtgcgctc tctgttccg accctgccgc ttaccggata	2100
	cctgtccgcc tttctccctt cgggaagcgt ggcgctttct catagctcac gctgtaggta	2160
	tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac ccccgttca	2220
30	gcccagaccgc tgcgccttat ccggttaacta tcgtcttgag tccaaccgg taagacacga	2280
	cttatcgcca ctggcagcag ccaactggtaa caggattagc agagcgaggt atgtaggcgg	2340
35	tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagga cagtatttgg	2400
	tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct cttgatccgg	2460
	caaacaaacc accgctggta gcggtgggtt ttttgtttgc aagcagcaga ttacgcgcag	2520
40	aaaaaaagga tctcaagaag atcctttgat cttttctacg gggctctgacg ctcagtggaa	2580
	cgaaaactca cgtaaggga ttttgggtcat gagattatca aaaaggatct tcacctagat	2640
45	ccttttaaat taaaaatgaa gttttaaatc aatctaaagt atatatgagt aaacttggtc	2700
	tgacagttac caatgcttaa tcagtgaggc acctatctca gcgatctgtc tatttcgttc	2760
	atccatagtt gcctgactcc ccgtcgtgta gataactacg atacgggagg gcttaccatc	2820
50	tggccccagt gctgcaatga taccgcgaga cccacgctca ccggctccag atttatcagc	2880
	aataaaccag ccagccggaa gggccgagcg cagaagtggc cctgcaactt tatccgcctc	2940
55	catccagtct attaattgtt gccgggaagc tagagtaagt agttcgccag ttaatagttt	3000
	gcgcaacggt gttgccattg ctacaggcat cgtgggtgtca cgctcgtcgt ttgggtatggc	3060
	ttcattcagc tccggttccc aacgatcaag gcgagttaca tgatccccca tgttgtgcaa	3120

```

    aaaagcgggtt agtcctctcg gtcctccgat cgttggtcaga agtaagttgg ccgcagtggt 3180
5   atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat ccgtaagatg 3240
    cttttctgtg actgggtgagt actcaaccaa gtcattctga gaatagtgtg tgcggcgacc 3300
    gagttgctct tgcccggcgt caatacggga taataccgcg ccacatagca gaactttaaa 3360
10  agtgctcatc attggaaaac gttcttcggg gcgaaaactc tcaaggatct taccgctgtt 3420
    gagatccagt tcgatgtaac ccactcgtgc acccaaactga tcttcagcat cttttacttt 3480
    caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa agggaataag 3540
15  ggcgacacgg aaatgttgaa tactcatact cttccttttt caatattatt gaagcattta 3600
    tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa ataaacaaat 3660
20  aggggttccg cgcacatttc cccgaaaagt gccac 3695

```

```

    <210> 22
    <211> 6640
25  <212> DNA
    <213> artificial

    <220>
    <223> vector vEM32 (7.5)
30

```

```

    <220>
    <221> misc_feature
    <222> (338)..(1263)
35  <223> Flank1 Del3

```

```

    <220>
    <221> gene
    <222> (1283)..(1962)
40  <223> Azami green

```

```

    <220>
    <221> misc_feature
    <222> (2002)..(1963)
45  <223> Ps

```

```

    <220>
    <221> misc_feature
    <222> (2009)..(2048)
50  <223> Ps

```

```

    <220>
    <221> gene
    <222> (2049)..(2447)
55  <223> BsdR

```

```

    <220>
    <221> promoter
    <222> (2454)..(2493)

```

```

<223> Ps promoter

<220>
<221> misc_feature
5 <222> (2494)..(2534)
   <223> TetO2

<220>
<221> gene
10 <222> (2535)..(3383)
   <223> DNase

<220>
<221> misc_feature
15 <222> (3414)..(3476)
   <223> p7.5

<220>
<221> gene
20 <222> (3533)..(4189)
   <223> TetR

<220>
<221> misc_feature
25 <222> (4196)..(4706)
   <223> Flank2 Del3

<220>
<221> gene
30 <222> (5655)..(6512)
   <223> AmpR

<400> 22
35 ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc 60
   attttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
   gataggggttg agtgttggtc cagtttgga caagagtcca ctattaaaga acgtggactc 180
40 caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
   ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaaggag 300
   ccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatggtgt 360
45 tattgtatac tgtctaaacg cgtagtaaaa acatggcgag gaaataaatc atataaaaaa 420
   tgatttcatg attaaaccat gttgtgaaaa agtcaagaac gttcacattg gcggacaatc 480
50 taaaaacaat acagtgattg cagatttgcc atatatggat aatgcggtat ccgatgtatg 540
   caattcactg tataaaaaaga atgtatcaag aatatccaga tttgctaatt tgataaagat 600
   agatgacgat gacaagactc ctactggtgt atataattat tttaaaccta aagatgccat 660
55 tcctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga 720
   taaagcgtgt gcgtgtatag agttaaatc atataaagta gccattcttc ccatggatgt 780

```

	ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga	840
	tgccggcacct ctcttaagaa gtgtaaccga taataatggt attatatcta gacaccagcg	900
5	tctacatgac gagcttccga gttccaattg gttcaagttt tacataagta taaagtccga	960
	ctattgttct atattatata tgggtgttga tggatctgtg atgcatgcaa tagctgataa	1020
10	tagaacttac gcaaataatta gcaaaaatat attagacaat actacaatta acgatgagtg	1080
	tagatgctgt tatTTTgaac cacagattag gattcttgat agagatgaga tgctcaatgg	1140
	atcatcgtgt gatatgaaca gacattgtat tatgatgaat ttacctgatg taggcgaatt	1200
15	tggatctagt atgttgggga aatatgaacc tgacatgatt aagattgctc tttcgggtggc	1260
	tggcacatcg gtggcgcccg cttacttggc ctgactcggc agcatagaat agcgagcaac	1320
20	ggcattctca tagagcttga ccttgttgtg atctttgtca tgcttcaaaa tctcaatgcg	1380
	gtggtccaca aagtgatagt ctggcacaacg gacatccttc tttgctttgt aagtagtttt	1440
	gaaatcacat cgataatggc cacctccttc aagcaacaga gccatgttaa catcaccctt	1500
25	cagcactcca tcacgtacgt acattttctc agtggatggc tcccatttaa gagtcttctt	1560
	ctgcataacc ggaccattgg gaggaaaagt cacaccatca aaacgaatgt catagaaaaa	1620
30	acagtgcgcc ctcatgctta tgttgcctgt ggcggtgcaa atgccctggc cttcataagt	1680
	catgcttctt tcccagtgat acccctcagg aaaagtctgc ttgaaatagt cctgaatatc	1740
	tgtcgggtac ttggtgaatg ccctgttgcc gtactggaac actgttgtca agatatcgta	1800
35	agcgaaaggc agaggtgcgc cttcagtgac gttcaggtct aaaatctgcg ttccctcgta	1860
	aggatttctt tttccttctc cttcaatcac gaaattatgc ccgtttacag tgccctctcat	1920
40	acacagcttg atcttcatct ctggtttaat cacactcacc attatttata ttccaaaaaa	1980
	aaaaaataaa atttcaattt ttgccggcaa aaattgaaat tttatttttt ttttttggaa	2040
	tataaataat ggccaagcct ttgtctcaag aagaatccac cctcattgaa agagcaacgg	2100
45	ctacaatcaa cagcatcccc atctctgaag actacagcgt cgccagcgca gctctctcta	2160
	gcgacggccg catcttcact ggtgtcaatg tatatcattt tactggggga ccttgtgcag	2220
50	aactcgtggc gctgggcact gctgctgctg cggcagctgg caacctgact tgtatcgtcg	2280
	cgatcggaaa tgagaacagg ggcatcttga gcccctgcgg acggtgccga caggtgcttc	2340
	tcatctgca tcttgggatc aaagccatag tgaaggacag tgatggacag ccgacggcag	2400
55	ttgggattcg tgaattgctg ccctctggtt atgtgtggga gggctaaact agtaaaaatt	2460
	gaaattttat tttttttttt tggaatataa atatccctat cagtgataga gatctcccta	2520
	tcagtgatag agatatgagg ggcatgaagc tgctgggggc gctgctggca ctggcgggccc	2580

	tactgcaggg ggccgtgtcc ctgaagatcg cagccttcaa catccagaca tttggggaga	2640
5	ccaagatgtc caatgccacc ctcgtcagct acatttgtgca gatcctgagc cgctatgaca	2700
	tgcacctggt ccaggagggtc agagacagcc acctgaactgc cgtggggaag ctgctggaca	2760
	acctcaatca ggatgcacca gacacctatc actacgtggt cagtgaacca ctgggacgga	2820
10	acagctataa ggagcgctac ctgttcgtgt acaggcctga ccagggtgtct gcggtggaca	2880
	gctactacta cgatgatggc tgcgagccct gcggaacga caccttcaac cgagagccag	2940
15	ccattgtcag gttcttctcc cggttcacag aggtcaggga gtttgccatt gttcccctgc	3000
	atgcggcccc gggggacgca gtagccgaga tcgacgctct ctatgacgtc tacctggatg	3060
	tccaagagaa atggggcttg gaggacgtca tgttgatggg cgacttcaat gcgggctgca	3120
20	gctatgtgag accctcccag tggtcaccca tccgcctgtg gacaagcccc accttccagt	3180
	ggctgatccc cgacagcgct gacaccacag ctacaccac gcactgtgcc tatgacagga	3240
25	tcgtggttgc agggatgctg ctccgaggcg ccgttggtcc cgactcggct cttcccttta	3300
	acttccaggc tgcttatggc ctgagtgaac aactggccca agccatcagt gaccactatc	3360
	cagtggaggt gatgctgaag taggagctcc accgcggtgg cggccgctct agaataaata	3420
30	ataaatacaa taattaatct ctcgtaaaag tagaaaatat attctaattt attgcaggat	3480
	ccccgggct gcaggaattc gatatcaagc ttatcgatac cgtcgaccgg agatgtctag	3540
35	attagataaa agtaaagtga ttaacagcgc attagagctg cttaatgagg tcggaatcga	3600
	aggtttaaca acccgtaaac tcgcccagaa gctaggtgta gagcagccta cattgtattg	3660
	gcatgtaaaa aataagcggg ctttgctcga cgccttagcc attgagatgt tagataggca	3720
40	ccatactcac ttttgccctt tagaagggga aagctggcaa gattttttac gtaataacgc	3780
	taaaagtttt agatgtgctt tactaagtca tcgcgatgga gcaaaagtac atttaggtac	3840
45	acggcctaca gaaaaacagt atgaaactct cgaaaatcaa ttagcctttt tatgccaaca	3900
	aggtttttca ctagagaatg cattatatgc actcagcgct gtggggcatt ttactttagg	3960
	ttgcgtattg gaagatcaag agcatcaagt cgctaaagaa gaaagggaaa cacctactac	4020
50	tgatagtatg ccgccattat tacgacaagc tatcgaatta tttgatcacc aagggtgcaga	4080
	gccagccttc ttattcggcc ttgaattgat catatgcgga ttagaaaaac aacttaaattg	4140
55	tgaaagtggg tccgcgtaca gcggatcccg ggaattcaga tcttattaag ggcccggaaa	4200
	gttttatagg tagttgatag aacaaaatac ataattttgt aaaaataaat cactttttat	4260
	actaatatga cacgattacc aatacttttg ttactaatat cattagtata cgctacacct	4320

	tttcttcaga catctaaaaa aataggtgat gatgcaactt tatcatgtaa tcgaaataat	4380
	acaaatgact acgttggttat gagtgccttgg tataaggagc ccaattccat tattctttta	4440
5	gctgctaaaa gcgacgtctt gtattttgat aattatacca aggataaaat atcttacgac	4500
	tctccatagc atgatctagt tacaactatc acaattaaat cattgactgc tagagatgcc	4560
10	ggtacttatg tatgtgcatt ctttatgaca tcgcctacaa atgacactga taaagtagat	4620
	tatgaagaat actccacaga gttgattgta aatacagata gtgaatcgac tatagacata	4680
	atactatctg gatctacaca ttcaccggaa actagggctt cctcgctcac tgactcgctg	4740
15	cgctcggtcg ttcggtcgcg gcgagcggta tcagctcact caaaggcggc aatacggtta	4800
	tccacagaat caggggataa cgcaggaaag aacatgtgag caaaaggcca gcaaaggcc	4860
20	aggaaccgta aaaaggccgc gttgctggcg tttttccata ggctccgccc cctgacgag	4920
	catcacaaaa atcgacgctc aagtcagagg tggcgaaaacc cgacaggact ataaagatac	4980
	caggcgtttc cccctggaag ctccctcgctg cgctctctctg ttccgaccct gccgcttacc	5040
25	ggatacctgt ccgcctttct cccttcggga agcgtggcgc tttctcatag ctcacgctgt	5100
	aggtatctca gttcggtgta ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccc	5160
30	gttcagcccg accgctgcgc cttatccggt aactatcgtc ttgagtccaa cccggtaaga	5220
	cacgacttat cgccactggc agcagccact ggtaacagga ttagcagagc gaggtatgta	5280
	ggcggtgcta cagagttctt gaagtgggtg cctaactacg gctacactag aaggacagta	5340
35	tttggtatct gcgctctgct gaagccagtt accttcggaa aaagagttgg tagctcttga	5400
	tccggcaaac aaaccaccgc tggtagcggg ggtttttttg tttgcaagca gcagattacg	5460
40	cgcagaaaaa aaggatctca agaagatcct ttgatctttt ctacgggggc tgacgctcag	5520
	tggaaacgaaa actcacgtta agggattttg gtcatgagat tatcaaaaag gatcttcacc	5580
	tagatccttt taaattaaaa atgaagtttt aaatcaatct aaagtatata tgagtaaact	5640
45	tggctctgaca gttaccaatg cttaatcagt gaggcaccta tctcagcgat ctgtctattt	5700
	cgttcatcca tagttgcctg actccccgtc gtgtagataa ctacgatacg ggagggctta	5760
50	ccatctggcc ccagtgtgc aatgataccg cgagaccac gctcaccggc tccagattta	5820
	tcagcaataa accagccagc cggaagggcc gagcgcagaa gtggctcctgc aactttatcc	5880
	gcctccatcc agtctattaa ttgttgccgg gaagctagag taagtagttc gccagttaat	5940
55	agtttgcgca acgttggtgc cattgctaca ggcacgtgg tgtcacgctc gtcgtttggg	6000
	atggcttcat tcagctccgg ttcccaacga tcaaggcgag ttacatgata ccccatgttg	6060
	tgcaaaaaag cggttagctc cttcggctct ccgacgttg tcagaagtaa gttggccgca	6120

```

gtgttatcac tcatggttat ggcagcactg cataattctc ttactgtcat gccatccgta 6180
5 agatgctttt ctgtgactgg tgagtactca accaagtcac tctgagaata gtgtatgcgg 6240
cgaccgagtt gctcttgccc ggcgtcaata cgggataata ccgcgccaca tagcagaact 6300
ttaaaagtgc tcatcattgg aaaacgttct tcggggcgaa aactctcaag gatcttaccg 6360
10 ctgttgagat ccagttcgat gtaaccact cgtgcaccca actgatcttc agcatctttt 6420
actttcacca gcgtttctgg gtgagcaaaa acaggaaggc aaaatgccgc aaaaaagga 6480
15 ataagggcga cacggaaatg ttgaatactc ataactcttc tttttcaata ttattgaagc 6540
atztatcagg gttattgtct catgagcgga tacatatttg aatgtattta gaaaaataaa 6600
caaatagggg ttccgcgcac atttccccga aaagtgccac 6640

20 <210> 23
    <211> 6624
    <212> DNA
    <213> artificial
25 <220>
    <223> vector vEM33 (H5)

30 <220>
    <221> misc_feature
    <222> (338)..(1263)
    <223> Fliank1 Del3

35 <220>
    <221> gene
    <222> (1283)..(1962)
    <223> Azami green

40 <220>
    <221> misc_feature
    <222> (2002)..(1963)
    <223> Ps

45 <220>
    <221> misc_feature
    <222> (2009)..(2048)
    <223> Ps

50 <220>
    <221> gene
    <222> (2049)..(2447)
    <223> BsdR

55 <220>
    <221> promoter
    <222> (2454)..(2493)
    <223> Ps promoter

```

```

<220>
<221> misc_feature
<222> (2494)..(2534)
<223> TetO2
5
<220>
<221> gene
<222> (2535)..(3383)
<223> DNase
10
<220>
<221> promoter
<222> (3414)..(3483)
<223> H5 promoter
15
<220>
<221> gene
<222> (3517)..(4173)
<223> TetR
20
<220>
<221> misc_feature
<222> (4180)..(4690)
<223> Flank2 Del3
25
<220>
<221> gene
<222> (5639)..(6496)
<223> AmpR
30
<400> 23
ctaaattgta agcgttaata ttttggtaaa attcgcgtta aatttttgtt aaatcagctc 60
attttttaac caatagggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga 120
35 gataggggttg agtggtgttc cagtttgga caagagtcca ctattaaaga acgtggactc 180
caacgtcaaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc 240
40 ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaaggag 300
ccccgattt agagcttgac ggggaaccac tatttagttg gtggtcgcca tggatggtgt 360
tattgtatac tgtctaaacg cgtagtaaaa acatggcgag gaaataaatc atataaaaaa 420
45 tgatttcatg attaaaccat gttgtgaaaa agtcaagaac gttcacattg gcggacaatc 480
taaaaacaat acagtgattg cagatttgcc atatatggat aatgcggtat ccgatgtatg 540
50 caattcactg tataaaaaaga atgtatcaag aatatccaga tttgctaatt tgataaagat 600
agatgacgat gacaagactc ctactggtgt atataattat tttaaacctt aagatgccat 660
tcctgttatt atatccatag gaaaggatag agatgtttgt gaactattaa tctcatctga 720
55 taaagcgtgt gcgtgtatag agttaaatc atataaagta gccattcttc ccatggatgt 780
ttcctttttt accaaaggaa atgcatcatt gattattctc ctgtttgatt tctctatcga 840

```

	tgccggcacct	ctcttaagaa	gtgtaaccga	taataatggt	attatatcta	gacaccagcg	900
	tctacatgac	gagcttccga	gttccaattg	gttcaagttt	tacataagta	taaagtccga	960
5	ctattgttct	atattatata	tggttgttga	tggatctgtg	atgcatgcaa	tagctgataa	1020
	tagaacttac	gcaaataatta	gcaaaaatat	attagacaat	actacaatta	acgatgagtg	1080
	tagatgctgt	tattttgaac	cacagattag	gattcttgat	agagatgaga	tgctcaatgg	1140
10	atcatcggtg	gatatgaaca	gacattgtat	tatgatgaat	ttacctgatg	taggcgaatt	1200
	tggatctagt	atgttgggga	aatatgaacc	tgacatgatt	aagattgctc	tttcgggtggc	1260
15	tggcacatcg	gtggcggccg	cttacttggc	ctgactcggc	agcatagaat	agcgagcaac	1320
	ggcatttctca	tagagcttga	ccttggttga	atctttgtca	tgcttcaaaa	tctcaatgcg	1380
	gtgggtccaca	aagtgatagt	ctggcaaacg	gacatccttc	tttgctttgt	aagtagtttt	1440
20	gaaatcacat	cgataatggc	cacctccttc	aagcaacaga	gccatgttaa	catcacccctt	1500
	cagcactcca	tcacgtacgt	acattttctc	agtggatggc	tcccatttaa	gagtcttctt	1560
25	ctgcataacc	ggaccattgg	gaggaaagtt	cacaccatca	aaacgaatgt	catagaaaaa	1620
	acagtcgccc	ctcatgctta	tgttgcttgt	ggcggtgcaa	atgccctggg	cttcataagt	1680
	catgcttctt	tcccagtgat	acccctcagg	aaaagtctgc	ttgaaatagt	cctgaatatc	1740
30	tgctgggtac	ttggtgaatg	ccctgttgcc	gtactggaac	actgttggtca	agatatcgta	1800
	agcgaaaagg	agaggtgcgc	cttcagtgac	gttcaggtct	aaaatctgcg	ttccctcgta	1860
35	aggatttcct	tttccttctc	cttcaatcac	gaaattatgc	ccgtttacag	tgctctcat	1920
	acacagcttg	atcttcatct	ctggtttaat	cacactcacc	attatttata	ttccaaaaaa	1980
	aaaaaataaa	atttcaattt	ttgccggcaa	aaattgaaat	tttatttttt	ttttttggaa	2040
40	tataaataat	ggccaagcct	ttgtctcaag	aagaatccac	cctcattgaa	agagcaacgg	2100
	ctacaatcaa	cagcatcccc	atctctgaag	actacagcgt	cgccagcgca	gctctctcta	2160
45	gcgacggccg	catcttcact	ggtgtcaatg	tatatcattt	tactggggga	ccttgtgcag	2220
	aactcgtggg	gctgggcact	gctgctgctg	cggcagctgg	caacctgact	tgtatcgtcg	2280
	cgatcggaag	tgagaacagg	ggcatcttga	gcccctgcgg	acggtgccga	caggtgcttc	2340
50	tcgatctgca	tcttgggatc	aaagccatag	tgaaggacag	tgatggacag	ccgacggcag	2400
	ttgggattcg	tgaattgctg	ccctctggtt	atgtgtggga	gggctaaact	agtaaaaatt	2460
55	gaaattttat	tttttttttt	tggaatataa	atatccctat	cagtgataga	gatctcccta	2520
	tcagtgatag	agatatgagg	ggcatgaagc	tgctgggggc	gctgctggca	ctggcggccc	2580
	tactgcaggg	ggccgtgtcc	ctgaagatcg	cagccttcaa	catccagaca	tttggggaga	2640

	ccaagatgtc caatgccacc ctogtcagct acatttgtgca gatcctgagc cgctatgaca	2700
5	tcgccctggg ccaggagggtc agagacagcc acctgactgc cgtggggaag ctgctggaca	2760
	acctcaatca ggatgcacca gacacctatc actacgtggg cagttagcca ctgggacgga	2820
	acagctataa ggagcgctac ctgttcgtgt acaggcctga ccagggtgtct gcgggtggaca	2880
10	gctactacta cgatgatggc tgcgagccct gcgggaacga caccttcaac cgagagccag	2940
	ccattgtcag gttctttctcc cggttcacag aggtcagggg gtttgccatt gttcccttgc	3000
15	atgcggcccc gggggacgca gtagccgaga tcgacgctct ctatgacgtc tacctggatg	3060
	tccaagagaa atggggcttg gaggacgtca tgttgatggg cgacttcaat gcgggctgca	3120
	gctatgtgag acctcccag tggtcaccca tccgctgtg gacaagcccc accttccagt	3180
20	ggctgatccc cgacagcgct gacaccacag ctacaccac gcactgtgcc tatgacagga	3240
	tcgtggttgc agggatgctg ctccgaggcg ccgttggtcc cgactcggct cttcccttta	3300
25	acttccaggc tgcctatggc ctgagtgacc aactggccca agccatcagt gaccactatc	3360
	cagtggaggt gatgctgaag taggagctcc accgcggtgg cggccgctct agaaaaaat	3420
	gaaaataaat acaaagggtc ttgagggttg tgtaaattg aaagcgagaa ataatcataa	3480
30	attggatccc ccgggctgca ggaattcgat ctcgagatgt ctagattaga taaaagtaaa	3540
	gtgattaaca gcgcattaga gctgcttaat gaggtcggaa tcgaagggtt aacaaccctg	3600
35	aaactcggcc agaagctagg tgtagagcag cctacattgt attggcatgt aaaaaataag	3660
	cgggctttgc tcgacgcctt agccattgag atgttagata ggcaccatac tcaacttttgc	3720
	cctttagaag gggaaagctg gcaagatttt ttacgtaata acgotaaaag ttttagatgt	3780
40	gctttactaa gtcacgcga tggagcaaaa gtacatttag gtacacggcc tacagaaaaa	3840
	cagtatgaaa ctctcgaaaa tcaattagcc tttttatgcc aacaagggtt ttcactagag	3900
45	aatgcattat atgcactcag cgctgtgggg cattttactt taggttgctg attggaagat	3960
	caagagcatc aagtcgctaa agaagaaagg gaaacaccta ctactgatag tatgccgcca	4020
	ttattacgac aagctatcga attatttgat caccaagggtg cagagccagc cttcttattc	4080
50	ggccttgaat tgatcatatg cggattagaa aaacaactta aatgtgaaag tgggtccgcg	4140
	tacagcggat cccgggaatt cagatcttat taaggggccc gaaagtttta taggtagtgt	4200
55	atagaacaaa atacataatt ttgtaaaaat aaatcacttt ttataactaat atgacacgat	4260
	taccaatact tttgttacta atatcattag tatacgtac accttttcc cagacatcta	4320
	aaaaaatagg tgatgatgca accttatcat gtaatogaaa taatacaaat gactacgttg	4380

	ttatgagtgc ttggtataag gagcccaatt ccattattct tttagctgct aaaagcgacg	4440
	tcttgtatatt tgataattat accaaggata aaatatotta cgactctoca tacgatgac	4500
5	tagttacaac tatcacaatt aaatcattga ctgctagaga tgccggtact tatgtatgtg	4560
	cattctttat gacatcgect acaaagaca ctgataaagt agattatgaa gaatactcca	4620
10	cagagttgat tgtaaataca gatagtgaat cgactataga cataatacta tctggatcta	4680
	cacattcacc ggaaactagg gcttctctgc tcaactgactc gctgcgctcg gtcgttcggc	4740
	tgccggcgagc ggtatcagct cactcaaagg cggtaatagc gttatccaca gaatcagggg	4800
15	ataacgcagg aaagaacatg tgagcaaaag gccagcaaaa ggccaggaac cgtaaaaagg	4860
	ccgcgttgct ggcggttttcc cataggctcc gccccctga cgagcatcac aaaaatcgac	4920
20	gctcaagtca gaggtggcga aaccgcagcag gactataaag ataccaggcg tttccccctg	4980
	gaagctccct cgtgcgctct cctgttccga ccctgccgct taccggatac ctgtccgcct	5040
	ttctcccttc gggaagcgtg gcgctttctc atagctcacg ctgtagggtat ctgagttcgg	5100
25	tgtaggctgt tcgctccaag ctgggctgtg tgcacgaacc ccccgttcag cccgaccgct	5160
	gcgccttata cggtaaactat cgtcttgagt ccaaccgggt aagacacgac ttatcgccac	5220
30	tggcagcagc cactggtaac aggattagca gagcgaggta tgtaggcggt gctacagagt	5280
	tcttgaagtg gtggcctaac tacggctaca ctagaaggac agtatttgggt atctgcgctc	5340
	tgctgaagcc agttaccttc ggaaaaagag ttggtagctc ttgatccggc aaacaaacca	5400
35	ccgctggtag cgggtggtttt tttgtttgca agcagcagat tacgcgcaga aaaaaaggat	5460
	ctcaagaaga tcctttgatc ttttctacgg ggtctgacgc tcagtggaac gaaaactcac	5520
40	gttaagggat tttggtcatg agattatcaa aaaggatctt cacctagatc cttttaaat	5580
	aaaaatgaag ttttaaatca atctaaagta tatatgagta aacttgggtc gacagttacc	5640
	aatgcttaat cagtgaggca cctatctcag cgatctgtct atttcgttca tccatagttg	5700
45	cctgactccc cgtcgtgtag ataactacga tacgggaggg cttaccatct ggccccagtg	5760
	ctgcaatgat accgcgagac ccacgctcac cggctccaga tttatcagca ataaaccagc	5820
50	cagccggaag ggccgagcgc agaagtggtc ctgcaacttt atccgcctcc atccagtcta	5880
	ttaattgttg ccgggaagct agagtaagta gttcgccagt taatagtttg cgcaacgttg	5940
	ttgccattgc tacaggcatc gtgggtgtcac gctcgtcgtt tggatatggct tcattcagct	6000
55	ccggttccca acgatcaagg cgagttacat gatcccccat gttgtgcaaa aaagcggtta	6060
	gctccttcgg tcctccgatc gttgtcagaa gtaagttggc cgcagtgtta tcaactcatgg	6120
	ttatggcagc actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga	6180

	ctggtgagta ctcaaccaag tcattctgag aatagtgtat gcggcgaccg agttgctctt	6240
	gcccggcgtc aatacgggat aataccgggc cacatagcag aactttaaaa gtgctcatca	6300
5	ttggaaaacg ttcttcgggg cgaaaactct caaggatctt accgctgttg agatccagtt	6360
	cgatgtaacc cactcgtgca cccaactgat cttcagcatc ttttactttc accagcgttt	6420
10	ctgggtgagc aaaaacagga aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga	6480
	aatgttgaat actcatactc ttcttttttc aatattattg aagcatttat cagggttatt	6540
	gtctcatgag oggatacata tttgaatgta tttagaaaaa taaacaaata ggggttccgc	6600
15	gcacatttcc cggaaaagtg ccac	6624
20		

CLAIMS

1. A modified poxvirus vector comprising a reporter gene and a selection component comprising a selection gene whose expression is regulated by the tetracycline operator/repressor system located between a pair of flanking sequences allowing for homologous recombination.
2. The modified poxvirus vector of claim 1 wherein the reporter gene encodes a fluorescent protein.
3. The modified poxvirus vector of claim 1, wherein the selection component comprises a selection gene that inhibits or slows down poxvirus replication in the host cell or is cytotoxic to the host cell.
4. The modified poxvirus vector of claim 1 wherein the reporter gene and the selection component are located between a single pair of flanking sequences allowing for homologous recombination.
5. The modified poxvirus vector of claim 1 wherein the reporter gene and the selection component are located between more than one pairs of flanking sequences allowing for homologous recombination.
6. The modified poxvirus vector of claims 3 – 5, wherein the selection component is a gene coding for a DNase.
7. The modified poxvirus vector of claims 1 – 6, wherein the poxvirus is vaccinia virus or Modified Vaccinia Ankara virus.
8. A cell comprising the modified poxvirus vector of claims 1 – 7.
9. Use of the modified poxvirus vector of claims 1 – 7 or the cell of claim 8 to generate a recombinant poxvirus.
10. A method for generating a recombinant poxvirus capable of expressing a gene of interest comprising the steps of:
 - 1) infecting a permissive host cell with the modified poxvirus vector of claim 1 – 7; and
 - (2) transfecting said infected permissive host cell with a plasmid comprising said gene of interest under conditions that permit homologous recombination between said vector and said plasmid,

whereby a recombinant poxvirus is produced.

11. The method of claim 10, further comprising the step of:
enhancing the production of recombinant poxvirus by selecting against the presence of the selection component.
12. The method of claims 10 – 11, further comprising the step of:
separating permissive host cells comprising recombinant poxvirus from permissive host cells comprising the modified poxvirus vector that has not undergone homologous recombination with the vector comprising the gene of interest.
13. The method of claims 10 – 12, further comprising the step of:
using the permissive host cells comprising the recombinant virus for carrying out at least one further passage of the recombinant poxvirus in previously uninfected permissive host cells.
14. The method of claims 10 – 13, wherein the poxvirus is vaccinia virus or Modified Vaccinia Ankara virus.
15. A cell comprising a recombinant poxvirus generated by the method of claims 10 – 14.
16. Use of the method of claims 10 – 14 or the cell of claim 15 to generate a recombinant poxvirus.
17. A recombinant poxvirus generated using the method of claims 10 – 14 or the cell of claim 15.
18. The modified poxvirus of claims 1 – 7, the cell of claim 8 or 15 or the recombinant poxvirus according to claim 17 as a medicament.
19. The modified poxvirus of claims 1 – 7, the cell of claim 8 or 15 or the recombinant poxvirus according to claim 17 as a therapeutic or prophylactic vaccine for the treatment or prevention of cancer, influenza, hepatitis, AIDS, mumps, rabies, encephalitis, stomach or duodenal ulcers, malaria, sleeping sickness, lyme disease, reactive arthritis, pneumonia, leprosy, diphtheria, candidiasis and/or toxoplasmosis.
20. A nucleic acid or its complement having at least 70% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 20-23 .