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(54) **Titre : ELEMENTS ADN RECOMBINANTS POUR L'EXPRESSION DE PROTEINES RECOMBINANTES CHEZ DES INSECTES**
(54) **Title: RECOMBINANT DNA ELEMENTS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN INSECTS**

(57) **Abrégé/Abstract:**

Reagents and methods are provided that allow for an improved expression of a recombinant protein in an insect, More specifically, the introduction of recombinant DNA elements into an insect larva allows for the increased expression of a recombinant protein, an improvement of the correct folding of said protein and an increase in the survival rate after infection of the insect These recombinant DNA elements can be introduced, for example, into insect larvae via a recombinant baculovirus, which has incorporated said elements. The recombinant DNA elements include nucleic acids encoding transcriptional regulators, such as IE-0 and IE- 1, transcriptional, enhancer elements, such as the homologous region (hr) and promoters.



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(54) Title: BACULOVIRAL DNA ELEMENTS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN A HOST INSECT

(57) Abstract: Reagents and methods are provided that allow for an improved expression of a recombinant protein in an insect. More specifically, the introduction of recombinant DNA elements into an insect larva allows for the increased expression of a recombinant protein, an improvement of the correct folding of said protein and an increase in the survival rate after infection of the insect. These recombinant DNA elements can be introduced, for example, into insect larvae via a recombinant baculovirus, which has incorporated said elements. The recombinant DNA elements include nucleic acids encoding transcriptional regulators, such as IE-0 and IE-1, transcriptional, enhancer elements, such as the homologous region (hr) and promoters.



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RECOMBINANT DNA ELEMENTS FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN INSECTS

FIELD OF THE INVENTION

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The present invention may be included in the field of biotechnology and it covers nucleic acid sequences comprising, for example, promoters, homologous regions (*hr*) as enhancers, and sequences encoding transcriptional regulators, for example, the baculovirus *Ac-*ie-01** cDNA, or any combination thereof, which are able to increase the efficiency of recombinant protein expression, for example in insect larvae. Moreover, the present invention is also directed to the vectors themselves comprising the above mentioned nucleic acid sequences of the invention, insects infected, transformed, transduced or transfected with those sequences or vectors, and methods for producing proteins by using the aforesaid sequences, vectors or insects.

STATE OF THE ART

The baculovirus expression vector system (BEVS) is a well-established method for the production of recombinant proteins to be used as vaccines, therapeutic molecules or diagnostic reagents. With its potential for over-expression and rapid speed of development, BEVS is one of the most attractive choices for producing recombinant proteins for any purpose. The most employed baculovirus vector used in industry for recombinant protein expression is based on *Autographa californica* multinuclear polyhedrosis virus (AcMNPV) with *Spodoptera frugiperda* 9 (*Sf9*) or 21 (*Sf21*) insect cells as suitable expression hosts (1), as well as *Trichoplusia ni* (*T. ni*) insect larvae as living biofactories (2). Since the BEVS was developed in the 80's (3), hundreds of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins, have been successfully produced in baculovirus-infected insect cells.

Worldwide, about 70,000 tons of silk are produced annually in a process that converts a low-value substrate, the leaves of the mulberry tree, to a high-value protein-based product: silk. Insects are highly efficacious protein producers because of their accelerated metabolism. Lepidoptera such as *Bombyx mori* or

Trichoplusia ni, are two of the most used insects in biotechnology. They grow in size about 5000 times in less than 2 weeks and produce more than a kilometer of silk per insect. While a cell from a silk gland may produce about 80 µg protein/cell/day, the best mammalian cell culture systems produces only about 50 pg protein/cell/day. Concerns arising from potential contamination by adventitious agents (such as viruses or prions) of recombinant proteins obtained from cultured mammalian cells are substantially lower, if not totally absent, in the case of insect-derived products for human or animal use. Although transgenic silkworms have been generated to produce human therapeutic proteins (4), this technology has not yet been used to produce vaccine antigens. However, genetically modified baculoviruses (*Autographa californica* nuclear polyhedrosis virus and *Bombyx mori* nucleopolyhedrovirus) have been used to generate insect-derived vaccines (insectigens). Basically, the same baculoviruses used for antigen expression in insect cell cultures can be used to infect insect larvae. Upon infection, the recombinant antigen is accumulated in insect tissues and, after 3–4 days of infection, the larvae can be processed to obtain the recombinant antigen in quantities that can reach between several hundreds of µg to several milligrams per infected larva. Experimental vaccines against animal infectious diseases have been tested with very good results, even using nonpurified soluble antigens obtained from larva, without any side effects in animals after repetitive immunization protocols (5, 6, 7, 8). Other proteins with different applications have also been produced in insects as living biofactories, such as enzymes (9, 10), antibodies (11, 12), hormones (13, 14), cytokines (15, 16) and diagnostic proteins (17, 18, 19, 20). Most of these above mentioned proteins were processed correctly after synthesis, and their functional activities remained intact in soluble larval protein extracts. Insects as living biofactories constitute a promising alternative to conventional fermentation technologies and also to plant-derived proteins because of the production versatility, scalability, efficiency and speed of development.

Accelerating recombinant protein expression, so that protein expression takes place before the cellular machinery of insect larvae is severely impaired by the baculovirus infection, would be an important improvement of the BEVS. Late

expression, driven by the conventional strong virus promoters of *polyhedrin* (*polh*) or *p10* genes, has serious disadvantages in the foreign protein post-translational modifications. Baculovirus promoters that allow for earlier expression than the conventionally used *polh* or *p10* promoters have been
5 characterized and been used for heterologous protein production, but showed a reduced productivity (21).

Another possibility for improving the BEVS would be to increase preservation of cell integrity at late times post-infection by reducing the virus-induced cell death.
10 Reduction in the severe impairment of the insect cell machinery at late times post-infection caused by BEVS should not only increase the time frame for producing and accumulating recombinant proteins (secreted or not), but also allow more time for the folding of complex proteins or any post-translational modification of the produced proteins. Baculovirus-infected insect larvae
15 produce and accumulate recombinant proteins during the course of infection and in a virus dose-dependent manner. A prolonged survival of the infected larvae together with a certain protection to high infectious doses would dramatically increase the productivity of insects serving as living biofactories.

20 Some baculovirus DNA elements have been determined to be involved in the activation of late expression factor genes, which are necessary for virus propagation. One of them is the immediate early (ie) protein IE-1 and its splice variant IE-0 from *AcMNPV* (*Autographa californica* multinuclear polyhedrosis virus). Translation of the *AcMNPV* mRNAs encoded by *Ac-ie-01* cDNA results in
25 both IE-0 and IE-1 production due to internal translation initiation. Both are thought to be critical mediators of baculovirus gene expression due to their potency as transcriptional regulators (22). Synthesized very early during infection, *AcMNPV* IE-1 is a 67-kDa dimeric DNA-binding protein that stimulates transcription in plasmid transfection assays through the activity of its N-terminal
30 acidic domain (23, 24). IE-1 accumulates within the nucleus, where it is maintained through late times (25). Transactivation by IE-1 is enhanced by its binding as a homodimer to the baculovirus homologous region (*hr*) sequences, which function as transcriptional enhancers and origins of viral DNA replication.

AcMNPV immediate early protein IE-0 (74 kDa) is identical to IE-1 except for an additional 54 amino acid residues at its N-terminus. AcMNPV IE-0 is a 72.6-kDa 636 amino acid protein composed of 38 amino acids encoded by *orf141* (*exon0*), 16 amino acids encoded by the upstream nontranslated leader of *ie1*, and the entire 582 amino acid IE-1 protein. The final product is therefore identical to IE-1 except for the additional 54 amino acids fused to the N-terminus. Presumably due to their common sequences, IE-0 and IE-1 share biochemical activities, including *hr* enhancer binding and transcriptional regulation.

10

Given the lack of novel alternative promoters stronger than those commercially used (*polh* and *p10*) and of any alternative to implement long-term expression in the baculovirus system by reducing virus-induced cell damage, the present invention is focused on solving said problems by means of the incorporation of recombinant DNA elements in baculovirus expression cassettes. The present invention surprisingly shows that said expression cassettes, containing baculovirus transcriptional regulators, an enhancer homologous region (*hr*) sequence and a promoter or a combination of promoters are able to increase recombinant protein production to unprecedented levels in insect larvae. Additionally, the expression cassettes of the present invention also increase the survival rate of baculovirus-infected insect larvae at late times post-infection using high infectious doses, thus minimizing the pathogenicity effect in insects caused by the baculovirus infection. On the other hand, an improvement in the integrity of cell functions during baculovirus infection also contributes to the correct recombinant protein posttranslational processing in insect larvae.

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SUMMARY OF THE INVENTION

The present invention provides products and methods for the improved expression of recombinant proteins in baculovirus-infected insect larvae.

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The following items are preferred embodiments for allowing this improved expression:

1. Insect comprising a nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators, wherein the nucleic acid is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus.

2. Insect according to item 1, further comprising at least one recombinant homologous region (*hr*) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein.

3. Insect according to item 2, wherein the recombinant homologous region (*hr*) is the sequence indicated in SEQ ID NO: 27 (*hr1*).

4. Insect according to item 2 or 3, wherein the promoter operably linked to the homologous region (*hr*) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

5. Insect according to any of the items 1-4, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

6. Insect according to any of the items 1-5, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of items 1-5.

7. Insect according to any one of the items 1-6, wherein the insect is derived from the genus *Lepidoptera*.

8. Insect according to any of the items 1-7, wherein the insect is selected from the group consisting of *Trichoplusia ni*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Ascalapha odorata*, *Bombyx mori*, *Rachiplusia ni* and *Stigmene acrea*.

9. Insect according to any of the items 1-8, wherein the nucleic acid sequence(s) is introduced into the insect by a recombinant baculovirus, preferably *AcMNPV* or *BmNPV*.

10. Method for producing a recombinant protein that comprises the use of an insect of any of the items 1-9 and the extraction and purification of the recombinant protein by conventional means.

5 11. Method for producing a recombinant protein according to item 10, wherein the recombinant protein is selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone or diagnostic protein reagent.

10 12. Rearing, feeding or injection medium for an insect comprising the nucleic acid sequence(s) as specified in any of the items 1-6.

BRIEF DESCRIPTION OF THE FIGURES

15 **Figure 1:** Schematic representation of the baculovirus recombinant DNA elements of the invention, comprising four principal elements: a sequence encoding for transcriptional regulators (**A**; e.g. IE0 and IE1), which expression is driven by a promoter (**B**; e.g. *polh* or *pB2₉*); an enhancer homologous region (*hr*) sequence (**C**; e.g. *hr1*), upstream of the promoters (**D**; e.g. *p10*, *pB2₉p10* or *p6.9p10*) driving the expression of the foreign gene coding for the recombinant protein. The scheme shows the theoretical mechanism of interaction between the recombinant DNA elements of the present invention that results in the unprecedented overexpression of the recombinant protein.

25 **Figure 2:** Different strategies that result in the generation of recombinant baculoviruses by the "Bac-to-Bac®" cloning system (invitrogen™).

30 **Figure 3:** General scheme for the generation of cloning, donor and transfer vectors compatible with other commercial technologies used to generate recombinant baculoviruses.

Figure 4: A) Recombinant GFP production 96 hours post-infection in *Trichoplusia ni* insect larvae (fifth instar) inoculated with 500 or 50,000 PFUs of

a conventional baculovirus expressing the protein under the control of the *polh* promoter or by a baculovirus containing the baculovirus cassette of the invention *polh Ac-ie-01/hr1p6.9p10GFP*. **B)** Percentage of larvae surviving 96 hours post-infection with the same baculoviruses as in panel A and at two different infectious doses, i.e. 500 and 50,000 PFUs.

Figure 5: **A)** Percentage of larvae surviving 96 hours post-infection with the baculovirus containing the expression cassette of the present invention *polhAc-ie-01/hr1p6.9p10GFP* with respect to a conventional baculovirus (*polhGFP*) at different infectious doses. **B)** Insect biomass at the moment of infection with a conventional baculovirus (*polhGFP*) or with a baculovirus containing the expression cassette of the present invention (*polhAc-ie-01/hr1p6.9p10GFP*) and the recovered biomass 96 hours post-infection. The infectious dose was 5×10^4 PFUs.

Figure 6: **A)** Percentage of larvae surviving 96 hours post-infection using 5×10^4 PFUs as the infectious dose of the baculovirus overexpressing the *Ac-ie-01* cDNA under the *polh* promoter (*polhAc-ie-01*) or using a conventional baculovirus expressing GFP under the *polh* promoter (*polhGFP*). **B)** Insect biomass at the time of infection with the same baculoviruses as in panel A and the recovered biomass at 96 hours post-infection. The infectious dose was 5×10^4 PFUs.

Figure 7: Comparison of the recombinant GFP protein expressed by a conventional baculovirus under the control of the *polh* promoter (2) or by a baculovirus containing the expression cassette of the present invention *polhAc-ie-01/hr1p6.9p10GFP* (1). Extracts from larvae infected with 5×10^5 PFUs of every baculovirus were obtained 96 hours post-infection. **A)** Coomassie blue staining of the recombinant GFP protein. **B)** Cell integrity after baculovirus infection determined by tubulin detection with a specific antiserum. **C)** Western blot detection of recombinant GFP protein by using a specific anti-GFP serum.

Figure 8: Schematic representation of the preferred elements contained in the baculovirus expression cassettes of the invention, comprising encoding

sequences for transcriptional regulators, homologous regions (*hr*) enhancing the transcription induced by promoter(s) of a foreign gene encoding a recombinant protein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention improves the expression of recombinant proteins by means of the introduction of recombinant DNA elements into insects.

5

These recombinant DNA elements of the present invention are sequences that cause the expression of baculovirus transcriptional regulators above endogenous levels and optionally enhancer homologous regions (*hr*) and promoters operably linked to these aforementioned elements.

10

Furthermore, the recombinant DNA elements may form part of an expression cassette.

“Expression cassette” refers to a nucleic acid sequence that contains recombinant DNA elements, which control (e.g. the promoter) and/or are required (e.g. the gene itself) for gene expression. The expression cassette can be introduced in a recombinant vector or baculovirus.

The recombinant DNA elements may be incorporated in a single nucleic acid sequence, cloning vector, transfer vector, recombinant baculovirus or cell. However, they can also be present in different nucleic acid sequences, cloning vectors, transfer vectors or recombinant baculoviruses and be introduced into the same cell.

The present invention surprisingly shows that introduction into insect larvae of sequences that cause the expression of baculovirus transcriptional regulators above endogenous levels and optionally the introduction of an enhancer homologous region (*hr*) sequence, a promoter or a combination of promoters is able to increase the production of a recombinant protein to unprecedented levels. This indicates the usefulness of this system for the expression of recombinant proteins *in vivo*.

Additionally, the introduction of these recombinant DNA elements into insect larvae with baculoviruses increases the survival rate of the larvae late after infection, especially after using high virus doses for infection (maximizes the recovered amount of biomass, i.e. the productivity of the system), as compared to
5 larvae infected with a conventional baculovirus. The insect biomass recovered at the end of the production process is significantly increased as well.

Also, the integrity of the molecular cell machinery and cell morphology of said baculovirus-infected larvae is improved as compared to larvae infected with a
10 conventional baculovirus. An improvement in the integrity of cell functions during baculovirus infection also contributes to the correct post-translational processing of the recombinant protein.

Thus, one aspect of the invention relates to an insect that contains a nucleic acid
15 sequence that allows for the expression above endogenous levels of transcriptional regulators. In a preferred embodiment, the transcriptional regulators are IE-1, IE-0 and/or fragments thereof. In a further preferred embodiment, the insect is a transgenic insect.

20 "Transcriptional regulator" refers to a regulatory protein that has the ability to modulate the transcription of specific genes by, for example, binding to enhancer or repressor regions and/or recruiting further proteins that are involved in transcription.

25 IE-1 and its splice variant IE-0 are transcriptional regulators that are endogenously expressed during baculovirus infection. According to the present invention, IE-1, IE-0 and/or fragments thereof are recombinantly expressed to increase the total level of these proteins above endogenous levels. This can be achieved through, for example, introducing further copies of the endogenous
30 gene or manipulating the expression of the promoter of the endogenous gene. Further copies of the endogenous genes can be introduced as transgenes under the control of a suitable promoter such as *polh* or *pB2₉*.

The expression level of the proteins IE-1, IE-0 and/or fragments thereof can be determined at both the mRNA and at the protein level with methods conventionally known to the person skilled in the art, such as quantitative PCR and Western Blot analysis.

5

According to the invention, IE-1, IE-0 and fragments thereof are encoded by the nucleic acids of SEQ ID NO: 1 (also referred to as *Ac-ie-01*) to SEQ ID NO: 5. SEQ ID NO: 1 is the *Ac-ie-01* cDNA that encodes both IE-1 and IE-0, SEQ ID NO: 2 is the coding sequence (CDS) of IE-1 and SEQ ID NO: 3 is the CDS of IE-0. SEQ ID NO: 4
10 and 5 are the CDSs of the N-terminal domains of IE-1 and IE-0 respectively that retain the catalytic transcriptional regulator activity. The proteins that are encoded by SEQ ID NO: 2-5 are represented by SEQ ID NO: 6-9 respectively.

The present invention furthermore discloses variants of SEQ ID NO: 1-9 that are
15 or encode amino acids that are able to function as a transcriptional regulator. These variants are nucleic or amino acids whose nucleotide or amino acid sequence differs in one or more positions from these parental nucleic or amino acids, whereby differences might be additions, deletions and/or substitutions of nucleotides or amino acid residues.

20

Nucleic and amino acid sequences of the present invention shall be distinguished from other nucleic and amino acid sequences by their degree of sequence identity or similarity respectively as determined using EMBOSS Needle with the default parameters (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

25 Methods for the generation of such variants include random or site directed mutagenesis, site-saturation mutagenesis, PCR-based fragment assembly, DNA shuffling, homologous recombination *in vitro* or *in vivo*, and methods of gene-synthesis.

30 The sequence of the variants of SEQ ID NO: 1-5 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% identical to the sequences of SEQ ID NO: 1-5.

The sequence of the variants of SEQ ID NO: 6-9 is at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% similar to the sequences of SEQ ID NO: 6-9.

The insect is preferably a lepidopter and more preferably an insect selected from the group consisting of *Trichoplusia ni*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Ascalapha odorata*, *Bombyx mori*, *Rachiplusia ni* and *Stigmene acrea*. In a preferred embodiment, the insect is a larva.

In a preferred embodiment, the insect of the present invention further contains, in addition to the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof, a recombinant homologous region (*hr*) that can enhance the expression of a recombinant protein by being operably linked to the respective promoter.

Homologous regions, *hr*, are comprised of repeated units of about 70-bp with an imperfect 30-bp palindrome near their center. Homologous regions are repeated at eight locations in the *AcMNPV* genome with 2 to 8 repeats at each side. Homologous regions have been implicated as both transcriptional enhancers and origins of baculovirus DNA replication.

“Enhancer region” refers to a control sequence, whose binding by transcriptional regulators increases the level of transcription of associated genes.

“Recombinant protein” refers to a protein that originates from recombinant DNA. Such proteins can be used for the benefit of humans and animals and may have industrial, commercial or therapeutic application.

“Being operably linked” refers to two nucleic acid sequences that are connected in a way that one influences the other in terms of, for example, transcriptional regulation.

“Promoter” refers to a DNA sequence to which RNA polymerase can bind to initiate transcription. The sequence may further contain binding sites for various proteins that regulate transcription, such as transcription factors. The promoter sequence may be composed of different promoter fragments (either different or the same fragments) that are localized closely in the DNA sequence and may be separated by linkers or spacer. Such promoters are referred to as chimeric promoters.

10 The enhancer homologous region sequence *hr* upstream of the promoter/s is preferably *hr1* (SEQ ID NO: 27). The promoters that drive the expression of the recombinant protein are preferably selected from the group comprising SEQ ID NO: 10-16 or a sequence that is able to function as a promoter and has at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% identity with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

In a preferred embodiment, the nucleic acid sequence comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

As indicated above, the recombinant promoters, sequences encoding transcriptional regulators and enhancer regions of the present invention do not need to form part of a single molecule, instead these sequences may form part of distinct molecules as long as they are operably linked, i.e. contained within the same cells.

The insect of the present invention preferably comprises a nucleic acid sequence encoding a recombinant protein. This nucleic acid sequence is operably linked to the nucleic acid sequence that allows for the expression above endogenous levels of the proteins IE-1, IE-0 and/or fragments thereof and optionally to a homologous region (*hr*).

The above described recombinant DNA elements are preferably introduced into the insect by a recombinant baculovirus. Preferably, the baculovirus is *AcMNPV* or *BmNPV* and the insect an insect larva. The baculovirus is administered to the larva by oral administration (*per os*) or more preferably by injection. In a further embodiment the insect is infected, transfected, transduced or transformed with the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of the present invention. Preferably, the insect larvae are reared in a rearing module, such as described in the patent application ES 2 232 308.

10 In a further aspect, the invention discloses methods for producing a recombinant protein using the insect of the present invention. To this end, an insect can be infected, transfected, transduced or transformed with the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of the present invention. After expression of the recombinant protein, extraction and
15 purification of the recombinant protein is done by conventional means.

In a preferred embodiment for protein production, the larvae are infected by injecting a high virus dose (higher than 10^4 Plaque Forming Units) of the recombinant baculovirus of the invention. 3-4 days after infection, the infected
20 larvae are processed and the whole soluble protein extract is obtained by the use of appropriate extraction buffers. Extracts are centrifuged and the lipid fraction eliminated. Then, the recombinant protein is purified by conventional means.

The recombinant protein that is preferably produced by the methods of the present invention is a protein selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic
25 protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone or diagnostic protein reagent.

30 One aspect of the invention relates to the use of the recombinant baculovirus, transfer vector, cloning vector or nucleic acid sequence of the invention in a rearing, feeding or injection medium for an insect.

The present invention discloses a baculovirus that can be used to produce the insect of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof.

5 “Baculovirus” refers to a family of infectious viruses for invertebrates, mainly infecting insects and arthropods. A “recombinant baculovirus” has further introduced recombinant DNA through, for example, homologous recombination or transposition. The recombinant baculovirus preferably originates from *AcMNPV* (*Autographa californica* nuclear polyhedrosis virus) or *BmNPV* (*Bombyx*
10 *mori* nucleopolyhedrovirus).

“Recombinant DNA” refers to a form of artificial DNA that is engineered through the combination or insertion of one or more DNA strands, thereby combining DNA that would normally not occur together.

15

“Recombinant DNA element” refers to a functional element within recombinant DNA, such as a promoter, enhancer or a gene. As mentioned above, the recombinant DNA elements of the present invention are sequences that cause the expression of baculovirus transcriptional regulators above endogenous
20 levels, enhancer homologous regions (*hr*) and promoters operably linked to these aforementioned elements. Preferably, the baculovirus transcriptional regulators IE-1, IE-0 or fragments thereof are expressed above endogenous levels.

25 The recombinant baculovirus preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred combinations of these recombinant DNA elements are as described
30 above for the insects. Furthermore, the recombinant baculovirus preferably contains a nucleic acid sequence encoding a recombinant protein.

The present invention discloses a transfer vector that can be used to produce the insect and/or recombinant baculovirus of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, in addition to a sequence suitable for integration or
5 transposition in a baculovirus.

Transfer vectors generally permit the insertion of genetic information into a baculovirus.

10 The transfer vector preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred combinations of these recombinant DNA elements are as described above for the
15 insects.

In one preferred aspect, the transfer vector comprises a nucleic acid sequence encoding said recombinant protein, whereas in another preferred embodiment the transfer vector lacks such sequence.

20

In a preferred embodiment, the transfer vector is a bacmid.

"Bacmid" refers to a plasmid construct which contains the DNA sequence sufficient for generating a baculovirus when transfected into a cell.

25

In a further preferred embodiment, the transfer vector is derived from any of the commercially available baculovirus expression systems "Bac-to-Bac®" (invitrogen™), "BacPAK™" (Clontech™), "FlashBAC™" (Oxford Expression Technologies™), "BacuVance™" (GenScript™), "Bac-N-Blue DNA™" (invitrogen™), "BaculoDirect™" (invitrogen™), "BacVector®" 1000, 2000, 3000 (Novagen®), "DiamondBac™" (Sigma-Aldrich®) or "BaculoGold™" (BD biosciences™).

30

The present invention discloses a cloning vector that can be used to produce the insect, recombinant baculovirus and/or transfer vector of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, which is further suitable for
5 bacterial replication.

“Cloning vector” refers to any vector that is suitable for cloning, which generally involves the presence of restriction sites, an origin of replication for bacterial propagation and a selectable marker.

10

The cloning vector preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred
15 combinations of these recombinant DNA elements are as described above for the insects.

In one preferred aspect, the cloning vector comprises a nucleic acid sequence encoding said recombinant protein (also referred to as the “donor vector”),
20 whereas in another preferred embodiment the cloning vector lacks such sequence.

The present invention discloses a nucleic acid sequence that can be used to produce the insect, recombinant baculovirus, transfer vector and/or cloning
25 vector of the present invention and comprises said sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments thereof.

The nucleic acid sequence preferably contains in addition to (i) the sequence for expression above endogenous levels of the proteins IE-0, IE-1 and/or fragments
30 thereof, (ii) a recombinant homologous region (*hr*) linked to (iii) a suitable promoter for driving the expression of a recombinant protein. The preferred combinations of these recombinant DNA elements are as described above for the insects.

In one preferred aspect, the nucleic acid sequence comprises a nucleic acid sequence encoding said recombinant protein, whereas in another preferred embodiment the nucleic acid sequence lacks such sequence.

5

SUMMARY OF SEQUENCES

SEQ ID NO:	Name:
1	Complete <i>Ac-ie-01</i> cDNA
2	Protein coding sequence (CDS) of IE-1
3	CDS of IE-0
4	CDS of the IE-1 N-terminal domain
5	CDS of the IE-0 N-terminal domain
6	IE-1 protein
7	IE-0 protein
8	IE-1 N-terminal domain protein
9	IE-0 N-terminal domain protein
10	<i>polh</i> (promoter)
11	<i>p10</i> (promoter)
12	<i>pB2₉p10</i> (promoter)
13	<i>p6.9p10</i> (promoter)
14	<i>pB2₉</i> (promoter)
15	<i>pB2p10</i> (promoter)
16	<i>polhp10</i> (promoter)
17	<i>polhAc-ie-01/hr1p10</i>
18	<i>polhAc-ie-01/hr1pB2₉p10</i>
19	<i>polhAc-ie-01/hr1p6.9p10</i>
20	<i>pB2₉Ac-ie-01/hr1p10</i>
21	<i>pB2₉Ac-ie-01/hr1pB2₉p10</i>
22	<i>pB2₉Ac-ie-01/hr1p6.9p10</i>
23	<i>polhAc-ie-01/hr1polh</i>
24	<i>pB2₉Ac-ie-01/hr1polh</i>
25	<i>polhAc-ie-01/hr1polhp10</i>

26	<i>pB2₉Ac-ie-01/hr1polhp10</i>
27	<i>Homologous region enhancer hr1</i>
28	<i>polhAc-ie-01</i>
29	<i>polhGFP</i>

DEPOSITION OF MICROORGANISMS ACCORDING TO THE BUDAPEST TREATY

5 Plasmids were deposited in the Spanish Type Culture Collection (CECT) (www.cect.org); University of Valencia, Parc Científic Universitat de València; Catedrático Agustín Escardino, 9; 46980 Paterna (Valencia), Spain, with the accession number CECT 8031, on the date October 4, 2011.

10 EXAMPLES

Example 1. Overexpression of baculovirus transcriptional regulators potentiates the enhancer function of a homologous region *hr* functionally linked to a promoter increasing recombinant protein expression in a
15 baculovirus vector expression system (BEVS).

Immediate early viral proteins encoded by the *Ac-ie-01* cDNA, i.e. IE-1 and IE-0, from *AcMNPV* are potent transcriptional regulators in baculoviruses. Transactivation mediated by these proteins is enhanced by their binding as a
20 homodimer to the baculovirus homologous region (*hr*) sequences, which act as transcriptional enhancers. *AcMNPV* IE-1/IE-0 are 67-72 kDa dimeric DNA-binding proteins that stimulate transcription in plasmid transfection assays through the activity of their N-terminal acidic domain (7, 8). Synthesized very early during infection, IE-1 and IE-0 accumulate within the nucleus, where they
25 are maintained through late times. Using the dual plasmid pFastBac™ (invitrogen™), the *Ac-ie-01* cDNA was cloned under the control of the *polh* promoter. In the same plasmid, but in another locus, the GFP encoding gene was cloned downstream of the *hr1p6.9p10* chimeric promoter that was previously synthesized and contains the homologous region *hr1* fused to the promoters *p6.9*

and *p10*. A schematic representation of the resulting baculovirus expression cassette of the present invention and the putative function of the recombinant DNA elements is shown in **Figure 1**. The resulting plasmid was used to generate a recombinant baculovirus by the “Bac-to-Bac®” system (Invitrogen™). In parallel, a conventional baculovirus expressing the GFP protein under the control of *polh* promoter was generated by the same system.

The expression of GFP protein mediated by the different baculoviruses was studied by fluorimetry at 96 hours post-infection in *Trichoplusia ni* larvae using a low or high infectious dose (5×10^2 or 5×10^4 respectively). The expression level of GFP was increased in larval extracts by the baculovirus containing above expression cassette of the present invention by about 13 to more than 40% depending on the virus dose used (**Figure 4A**). Similar results were observed by using baculoviruses in which the *Ac-ie-01* cDNA was expressed under the control of the insect-derived *pB2₉* promoter or when the GFP was expressed under the control of the *hr1pB2₉p10* promoter (data not shown).

Example 2. The baculovirus expression cassettes of the invention increase the baculovirus-infected insect larvae surviving rates and insect biomass recovered using high infectious doses through the transcriptional regulators encoded by the *Ac-ie-01* cDNA

In the previous example an advantage of baculoviruses expressing the recombinant protein in the context of the baculovirus cassette of the present invention in terms of protein productivity was shown. However, the main difference observed was in respect to the percentage of surviving larvae at high infectious doses (maximum productivity). Under such infection conditions, the baculovirus containing the expression cassette of the present invention increased by about 70% the larvae survival rates (**Figure 4B**). This means that by using a conventional baculovirus the optimal infection conditions were using an infectious dose of 5×10^2 PFUs (maximum larvae surviving rate). In contrast, by using a baculovirus containing the expression cassette of the present invention, a dose of 5×10^4 could be used, recovering the same number of larvae

at the end of the production process (**Figure 4B**). Under such optimal production conditions (infection with 5×10^4 PFUs), the baculovirus containing the expression cassette of the present invention yields more than twice the amount of recombinant protein produced in insect larvae infected with the optimal dose
5 for the conventional baculovirus (5×10^2 PFUs) (**Figure 4 A and B**).

Larvae infection studies using different infectious doses of a conventional baculovirus (*polhGFP*) or a baculovirus containing the expression cassette of the present invention *polhAc-ie-01/hr1p6.9p10GFP*, both expressing the GFP protein,
10 revealed increasing surviving rates of infected larvae when the baculovirus containing the expression cassette of the present invention was used (**Figure 5A**). At the highest infectious dose (5×10^4) the survival rate of larvae infected with the expression cassette of the present invention increased by more than 70% as compared to larvae infected with a conventional baculovirus. This
15 increase in infected larvae surviving rates had direct dramatic consequences in the insect biomass recovered at the end of the production process, with a 80% increase when the baculovirus containing the expression cassette of the present invention was used at the highest infectious dose (maximum productivity) (**Figure 5B**).

20

To determine the genetic element/s responsible for such interesting properties related to the increase of survival rates after infection with high doses of the baculovirus of the invention, a recombinant baculovirus expressing the transcriptional regulators encoded by the *Ac-ie-01* cDNA under the control of the
25 *polh* promoter was generated. Then, *T. ni* larvae were infected with a high infectious dose (5×10^4 PFUs) of this baculovirus. As control, we used a baculovirus expressing the GFP protein under the control of the same promoter. Similarly to the larvae infected with the expression cassette of the present invention *polhAc-ie-01/hr1p6.9p10GFP*, larvae infected with the baculovirus
30 overexpressing the *Ac-ie-01* cDNA (*polhAc-ie-01*) also showed increased survival rates when compared with larvae infected with a conventional baculovirus expressing the GFP reporter protein under the control of the same promoter (*polhGPF*) (**Figure 6A**). This strongly suggests that the overexpression of the

transcriptional regulators used in the baculovirus expression cassette of the present invention protects the insect larvae from the baculovirus-induced mortality, allowing long-term expression (more recombinant protein production) and increasing the insect biomass recovery using high infectious
5 doses (maximum productivity) (**Figure 6B**).

**Example 3. Overexpression in a baculovirus expression system of transcriptional regulators encoded by the *Ac-ie-01* cDNA facilitates the post-translational processing of recombinant proteins in infected insect
10 larvae used as biofactories.**

Cellular integrity during baculovirus infection is of great importance to ascertain the correct folding or any other post-translational modification of foreign proteins expressed by this system. The baculovirus strong promoters commonly
15 used for research and production, such as *polh* and *p10*, only express the foreign genes at late times post-infection when infected cells already show severe cytopathic effects and the cellular viability decreases. As described above, the overexpression of the transcriptional regulators used in the baculovirus expression cassette of the present invention protects cells from pathogenic
20 effects of the baculovirus infection, allowing a wide temporal window for recombinant protein production in cells remaining fully viable.

The relevance of the recombinant DNA elements incorporated into the expression cassette of the invention in relation to post-translational
25 modifications of recombinant proteins in insect larvae as living biofactories was studied. For this purpose, a conventional baculovirus expressing the reporter protein GFP under the control of the *polh* promoter and a baculovirus incorporating the baculovirus cassette of the present invention and also expressing the GFP protein (*polhAc-ie-01/hr1p6.9p10GFP*) were used to infect
30 insect larvae at a dose of 5×10^4 PFUs.

Infected larvae extracts were analysed at 96 hours post-infection by SDS-PAGE gels and Coomassie blue staining (**Figure 7A**). Interestingly, GFP protein

expressed by a conventional baculovirus showed a band with a reduced molecular weight (lower than predicted), suggesting degradation or misfolding of the recombinant protein. In contrast, when the GFP protein expression was mediated by the baculovirus expression cassette of the present invention, the GFP band presented the expected molecular weight of this protein, i.e. about 27 kDa.

The infected larvae extracts were also analyzed by Western blot using anti-GFP monoclonal antibody (mab2515; Millipore™) (**Figure 7C**). The GFP protein was detected in larvae extracts infected by both baculoviruses, but showed a difference in electrophoretic mobility of the recombinant protein as observed by Coomassie blue staining.

In parallel, the integrity of the cell machinery was measured at different times post-infection by Western blot analysis of the cellular tubulin protein using a specific antiserum (**Figure 7B**). Infection with a conventional baculovirus impaired severely the cell integrity at 96 hours post-infection since the tubulin band detected decreased dramatically after this time point (degradation as a result of a complete loss of cell integrity). Consistent with the cellular protection induced by the recombinant DNA elements contained in the baculovirus expression cassette of the invention, cellular tubulin was not equally affected in cells infected by the recombinant baculovirus engineered with the expression cassette.

Example 4. Cell culture and viruses.

The *Spodoptera frugiperda* Sf21 or Sf9 cell lines were cultured in 6-well tissue culture plates (1×10^6 cells/well) in TNM-FH insect medium (Pan Biotech™, Germany) containing 10 % heat-inactivated fetal bovine serum (Pan Biotech™, Germany) at 27°C. AcMNPV recombinant baculoviruses were obtained by the “Bac-to-Bac®” Baculovirus Expression System (invitrogen™, USA). Different transfer vectors containing the recombinant DNA elements of the invention were generated using the pFastBac™-DUAL plasmid (invitrogen™). The promoters and

regulatory elements incorporated into pFastBac™-DUAL were synthesized (GenScript™, USA) with the adequate flanking restriction sequences to facilitate the cloning. These transfer vectors were used to transfect *Sf21* cells with Cellfectin® (invitrogen™, USA). The resulting recombinant baculoviruses from the infection of *Sf21* cells were then passaged twice in cells and titered by the plaque assay method. The obtained gene constructs of the baculovirus expression cassettes are schematically shown in **Figure 8**, showing different potential combinations of promoters driving the expression of the *Ac-ie-01* cDNA or the foreign gene (e.g. GFP). The different expression cassettes were used to generate the recombinant baculoviruses used in the examples shown in **Figures 4 to 7**.

Example 5. Generation of the cloning vector

The cloning vector is a small piece of DNA containing the baculovirus expression cassette of the present invention into which a foreign DNA fragment can be inserted by treating the vehicle and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together. The essential characteristics of the cloning vector must include a synthetic multiple cloning site (MCS) to facilitate the insertion of foreign genes directed in a chosen orientation, a selectable marker, such as an antibiotic resistance to allow the selection of positively transformed cells and a functional origin of replication (ORI) for propagation in bacteria.

Example 6. Generation of the donor vector containing the baculovirus expression cassette of the present invention

A donor vector consists of a cloning vector, for example a pUC57 plasmid, containing the baculovirus expression cassette, into which a foreign gene has been cloned using the appropriate restriction enzymes. The baculovirus expression cassette of the present invention was synthesized by ligating the following DNA sequences: (i) the baculovirus transcriptional regulator encoding sequence *Ac-ie-01* downstream of a promoter sequence, such as the *polh* or the *pB2* promoter, and upstream of the HSV TK polyadenylation signal and (ii) in

another locus an enhancer sequence, for example, the homologous region *hr1*, upstream of (iii) a promoter sequence, for example, *pB2₉p10*, *p10*, *p6.9p10* or *polh*, followed by a multiple cloning site (MCS) for cloning the gene of interest and the SV40 polyadenylation signal downstream of the MCS (**Figure 1**). The
5 baculovirus expression cassette is flanked by specific restriction sites (for example *Bgl*III and *Bst*Z17I at the 5'-terminal end and *Bgl* II and *Sgf*I at the the 3'-terminal end) to facilitate subcloning into a transfer vector of a commercial baculovirus generation system (based on transposition, for example the "Bac-to-Bac[®]" system (invitrogen[™]), or based on homologous recombination, for
10 example "flashBAC[™]" (Oxford Expression Technologies[™]), "Baculogold[™]" (BD Biosciences[™]), "BacPAK6[™]" (Clontech[™]), "Bac-N-Blue DNA[™]" (invitrogen[™])) (**Figure 2 and 3**).

The encoding gene of the Green Fluorescence Protein (GFP) was cloned into the
15 MCS of the cloning vector using the *Nco* I and *Spe* I restriction sites, generating the donor plasmid vector (**Figure 2**).

Example 7. Generation of the transfer vector containing the baculovirus expression cassette of the present invention

20 The transfer vector was generated by digesting a donor vector with *Bst*Z17 I of the 5'-flanking site and with *Xba* I and cloning it into the transfer vector pFastBac[™]1 that was also digested with the same enzymes. In this case, as a result of the subcloning, the SV40 polyadenylation signal of the baculovirus
25 expression cassette is exchanged by the SV40 polyadenylation signal from the transfer vector. Apart from this, all the elements of the expression cassette are included in the pFastBac transfer vector, substituting the *polh* promoter and MCS of the original commercial transfer vector (**Figure 2**).

Example 8. Generation of the baculovirus expression vector containing the baculovirus expression cassette of the present invention using the “Bac-to-Bac®” system

5 The modified transfer vector pFastBac™1 and the individual baculovirus expression cassette were used to generate a recombinant baculovirus by using the “Bac-to-Bac®” Baculovirus Expression System. More specifically, the modified transfer vector was used to transform the *E. coli* host strain DH10Bac™ that contains a baculovirus shuttle vector (bacmid) and a helper plasmid, and allows
10 the generation of a recombinant bacmid following transposition of the expression cassette. The DNA of the recombinant bacmid containing the baculovirus expression cassette of the present invention and the GFP encoding gene was then used to transfect insect cells, for example, Sf21 cells, using Cellfectin®. 72 hours post-transfection, cells were harvested and the first
15 recombinant baculovirus generation was obtained (**Figure 2**). This recombinant baculovirus could then be further amplified and/or titered following conventional protocols. Similar procedures can be used to generate recombinant baculoviruses with other transfer vectors provided by commercial BEVSs (**Figure 3**).

20

Example 9. Rearing and infection of insect larvae.

Trichoplusia ni (cabbage looper) larvae were reared under level 2 biosafety conditions. Eggs were placed into specially designed larva developmental cages
25 containing an artificial insect diet and were kept in growth chambers at 22 °C under controlled humidity (50%) and light period (8 h/day) conditions.

Fifth-instar larvae (last instar larvae before pupation), were used for all infection experiments. The standard weight of each larva was approximately 120-130 mg
30 and larvae were injected near the proleg (forward of the body cavity) with 5 µl of recombinant baculoviruses diluted to reach the number of plaque forming units (PFU) per dose selected. Larvae were processed at 96 hpi. The larvae collected were frozen immediately to be stored at -20°C until they were processed for

recombinant protein quantification. Total soluble, non-denatured proteins (TSNDPs) from frozen *T. ni* larvae infected by the baculoviruses were obtained by homogenization using a Bag Mixer® blender (Interscience™, France) for 2 min. Extraction buffer was composed of PBS 1x, Triton X-100 at 0.01%, Complete
5 protease inhibitor cocktail (Roche™, Germany), and DTT 25 mM.

Example 10. Fluorimetric analysis.

About 20 µg of total soluble proteins derived from infected cells, containing
10 different amounts of recombinant GFP protein, were analyzed and quantified by a Tecan™ GENios™ (CA, USA) fluorescence plate reader (excitation [Ex], 485/emission [Em], 535).

Example 11. Western blot analysis.

15 Total soluble protein fractions (10 µg) from larvae infected with the recombinant baculoviruses were resolved in 15% SDS-PAGE gels. Gels were stained by the Coomassie blue staining method or transferred to nitrocellulose membranes. Western blots were probed with the anti-GFP monoclonal antibody mab2515
20 (Millipore™, USA) or tubulin antiserum (T5168; Sigma-Aldrich™) at 1:1000 and the immunocomplexes were revealed with anti-mouse IgG-horseradish peroxidase (HRP)-labeled conjugate (KPL™, UK), diluted 1:2,000 as a secondary antibody. Protein bands were detected using an ECL western blotting detection system and analyzed by the ChemiDoc™ XRS Gel Imaging System (Bio-Rad™,
25 USA).

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AMENDED CLAIMS

1. Insect comprising a nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection ~~of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators~~, wherein the nucleic acid is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus;

wherein the insect further comprises a nucleic acid sequence encoding a recombinant protein operably linked to the above nucleic acid sequence and a promoter that drives the expression of said recombinant protein, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

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(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16;

~~(c)~~ wherein the

~~Insect according to claim 1,~~ further comprising at least one recombinant homologous region (hr) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein,

wherein the insect is derived from the genus Lepidoptera, and

2. wherein the nucleic acid sequence(s) is introduced into the insect by a recombinant baculovirus.

3.2. Insect according to claim 2.1, wherein the recombinant homologous region (hr) is the sequence indicated in SEQ ID NO: 27 (hr1).

4.3: Insect according to claim 2.1 or 3.2, wherein the promoter operably linked to the homologous region (hr) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

5.4. Insect according to any of the claims 1-4.3, comprising a nucleic acid sequence that comprises combinations of recombinant

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promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

~~6-5.~~ Insect according to any of the claims 1-54, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of claims 1-54.

~~7. Insect according to any of the claims 1-6, wherein the insect is derived from the genus Lepidoptera.~~

~~8-6.~~ Insect according to any of the claims 1-75, wherein the insect is selected from the group consisting of *Trichoplusia ni*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Ascalapha odorata*, *Bombyx mori*, *Rachiplusia ni* and *Stigmene acrea*.

~~9-7.~~ Insect according to any of the claims 1-86, wherein the nucleic acid sequence(s) is introduced into the insect by a recombinant baculovirus, preferably selected from AcMNPV or BmNPV.

~~10-8.~~ Method for producing a recombinant protein that comprises the use of an insect of any of the claims 1-9-7 and the extraction and purification of the recombinant protein by conventional means.

~~11-9.~~ Method for producing a recombinant protein according to claim ~~108~~, wherein the recombinant protein is selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone or diagnostic protein reagent.

~~12. Rearing, feeding or injection medium for an insect comprising the nucleic acid sequence(s) as specified in any of the claims 1-6.~~

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AMENDED CLAIMS

1. Insect comprising a nucleic acid sequence that allows for the expression of the proteins IE-1, IE-0 and/or fragments thereof functioning as transcriptional regulators above endogenous levels obtained during baculovirus infection, wherein the nucleic acid is selected from the group consisting of:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 1-5;
- (b) a nucleic acid sequence having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 1-5 and encoding a protein able to function as a transcriptional regulator in a recombinant baculovirus;
- (c) a nucleic acid sequence encoding an amino acid containing the amino acid sequence indicated in any of SEQ ID NO: 6-9; and
- (d) a nucleic acid sequence encoding an amino acid sequence having a sequence similarity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the amino acid sequence indicated in any of SEQ ID NO: 6-9 and able to function as a transcriptional regulator in a recombinant baculovirus;

wherein the insect further comprises a nucleic acid sequence encoding a recombinant protein operably linked to the above nucleic acid sequence and a promoter that drives the expression of said recombinant protein, selected from the group of nucleic acids comprising:

- (a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and
- (b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a

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sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16;

wherein the insect further comprises at least one recombinant homologous region (hr) as enhancer region, operably linked to any promoter that is suitable for driving the expression of a recombinant protein,

wherein the insect is derived from the genus Lepidoptera, and

wherein the nucleic acid sequence(s) is introduced into the insect by a recombinant baculovirus.

2. Insect according to claim 1, wherein the recombinant homologous region (hr) is the sequence indicated in SEQ ID NO: 27 (hr1).

3. Insect according to claim 1 or 2, wherein the promoter operably linked to the homologous region (hr) is selected from the group of nucleic acids comprising:

(a) a nucleic acid containing the nucleotide sequence indicated in any of SEQ ID NO: 10-16; and

(b) a nucleic acid sequence able to function as a promoter in a recombinant baculovirus and having a sequence identity of at least 70%, preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% with the nucleotide sequence indicated in any of SEQ ID NO: 10-16.

4. Insect according to any of the claims 1-3, comprising a nucleic acid sequence that comprises combinations of recombinant promoters, sequences encoding transcriptional regulators and enhancer regions selected from the group comprising SEQ ID NO: 17-26.

5. Insect according to any of the claims 1-4, further comprising a nucleic acid sequence encoding a recombinant protein, wherein said

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nucleic acid sequence is operably linked to a nucleic acid sequence selected from the group consisting of the nucleic acid sequences of claims 1-4.

6. Insect according to any of the claims 1-5, wherein the insect is selected from the group consisting of *Trichoplusia ni*, *Spodoptera frugiperda*, *Spodoptera exigua*, *Ascalapha odorata*, *Bombyx mori*, *Rachiplusia ni* and *Stigmene acrea*.

7. Insect according to any of the claims 1-6, wherein the nucleic acid sequence(s) is introduced into the insect by a recombinant baculovirus selected from AcMNPV or BmNPV.

8. Method for producing a recombinant protein that comprises the use of an insect of any of the claims 1-7 and the extraction and purification of the recombinant protein by conventional means.

9. Method for producing a recombinant protein according to claim 8, wherein the recombinant protein is selected from the group comprising subunit monomeric vaccine, subunit multimeric vaccine, virus like particle, therapeutic protein, antibody, enzyme, cytokine, blood clotting factor, anticoagulant, receptor, hormone or diagnostic protein reagent.

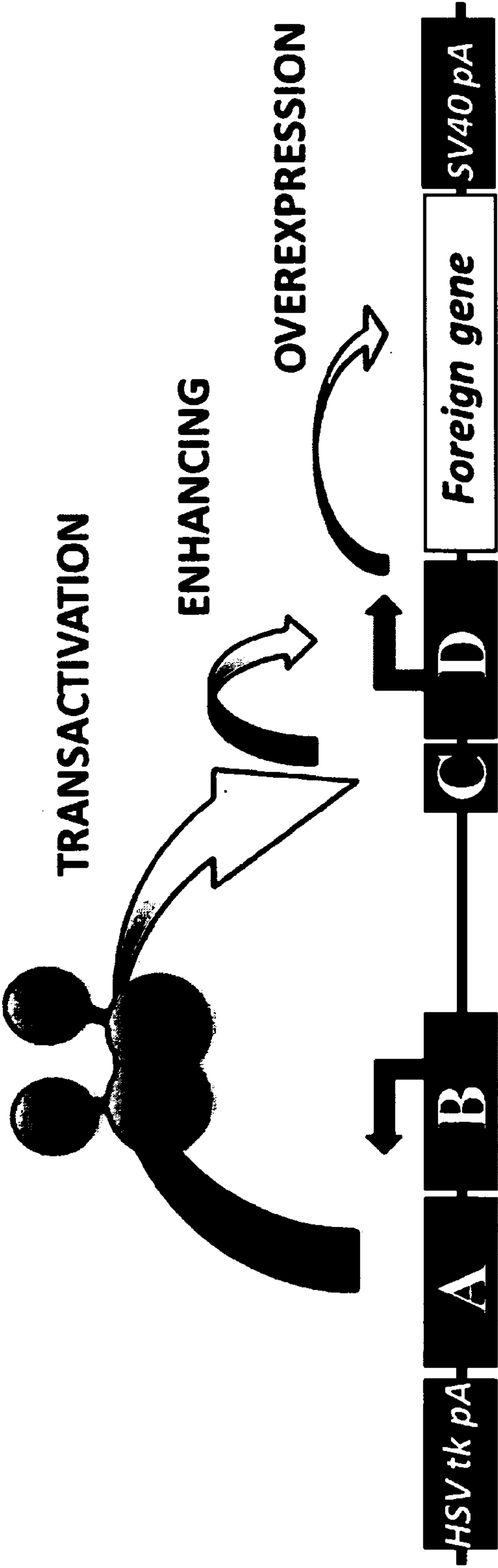


Figure 1

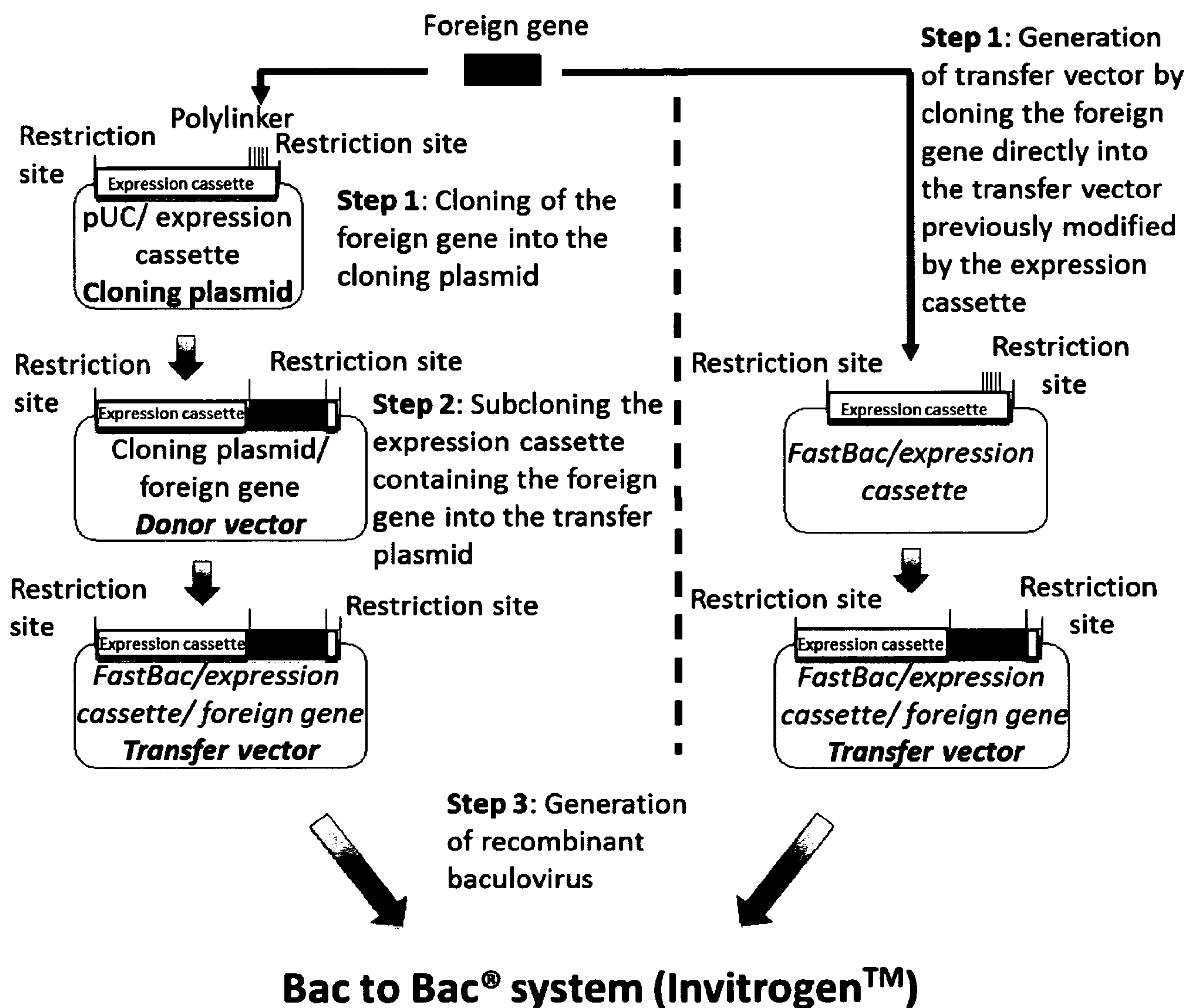
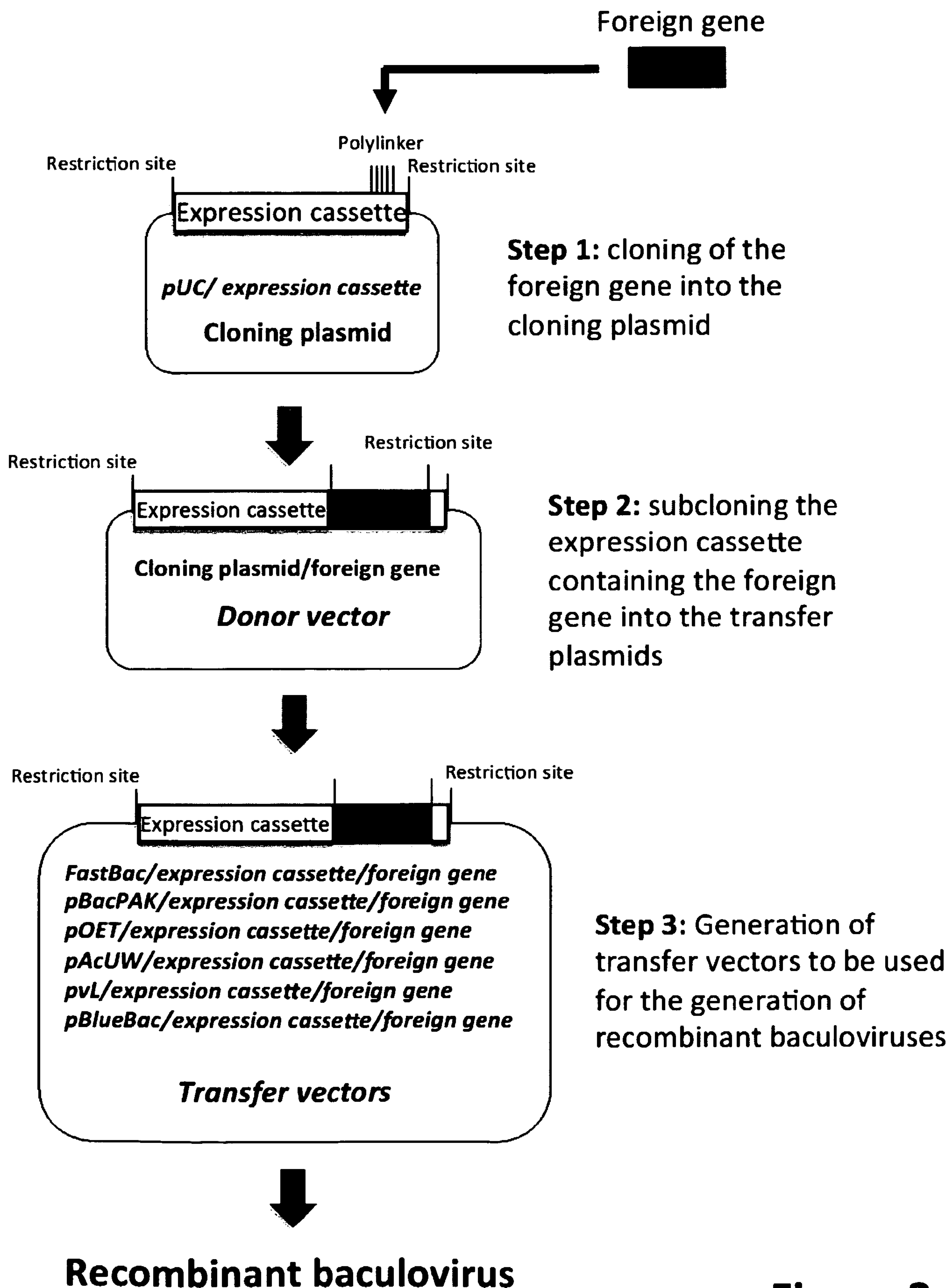


Figure 2

**Figure 3**

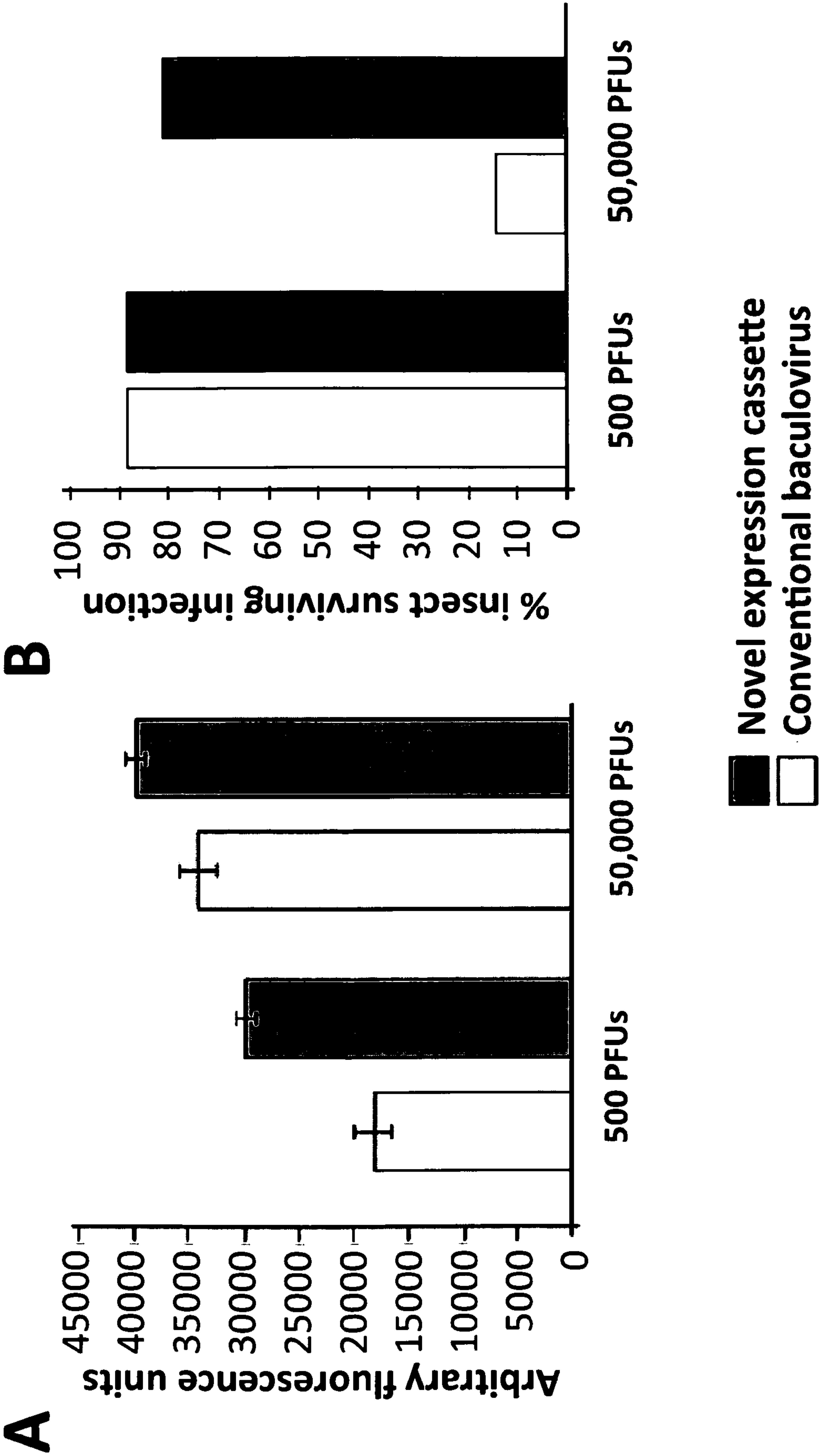


Figure 4

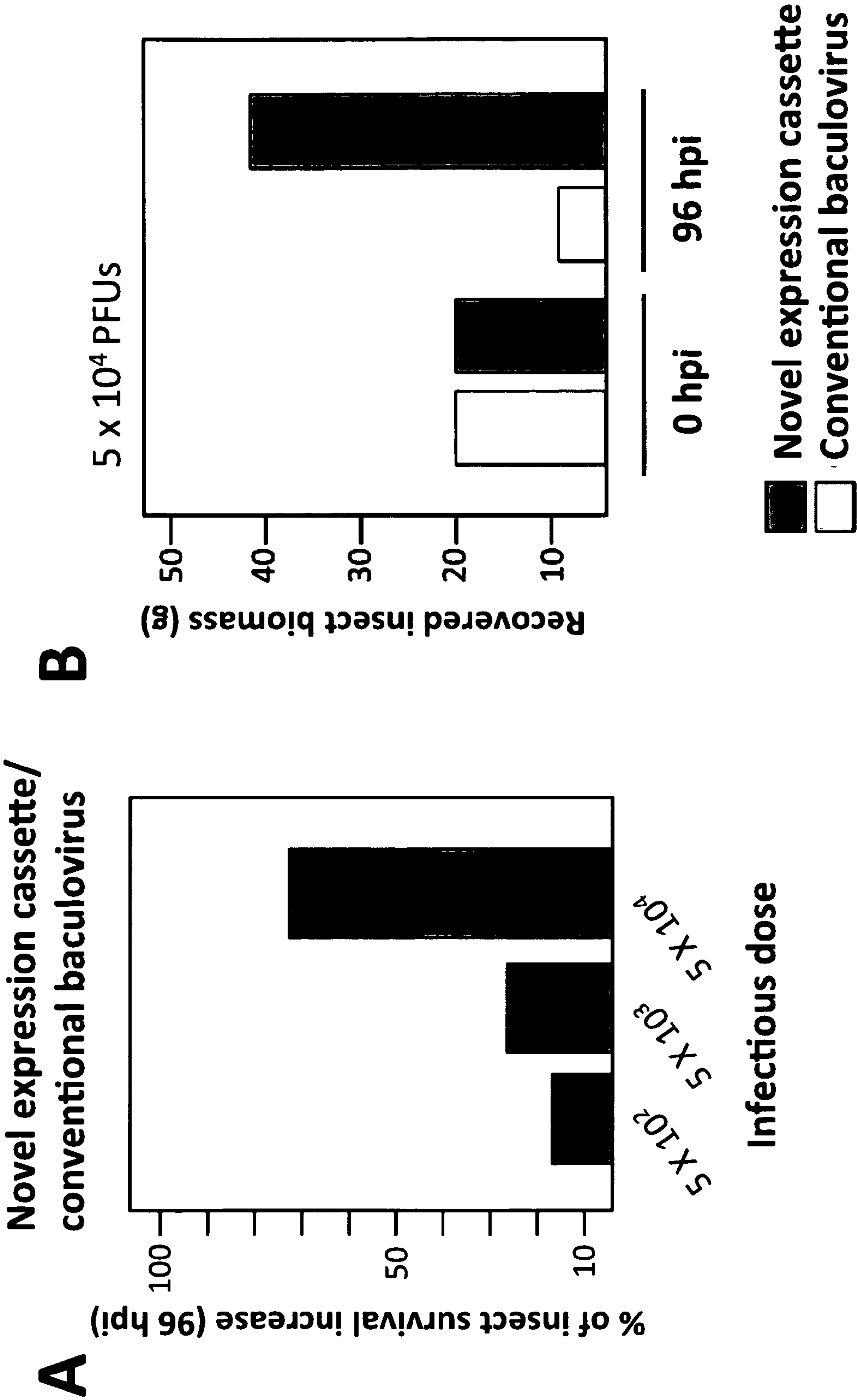


Figure 5

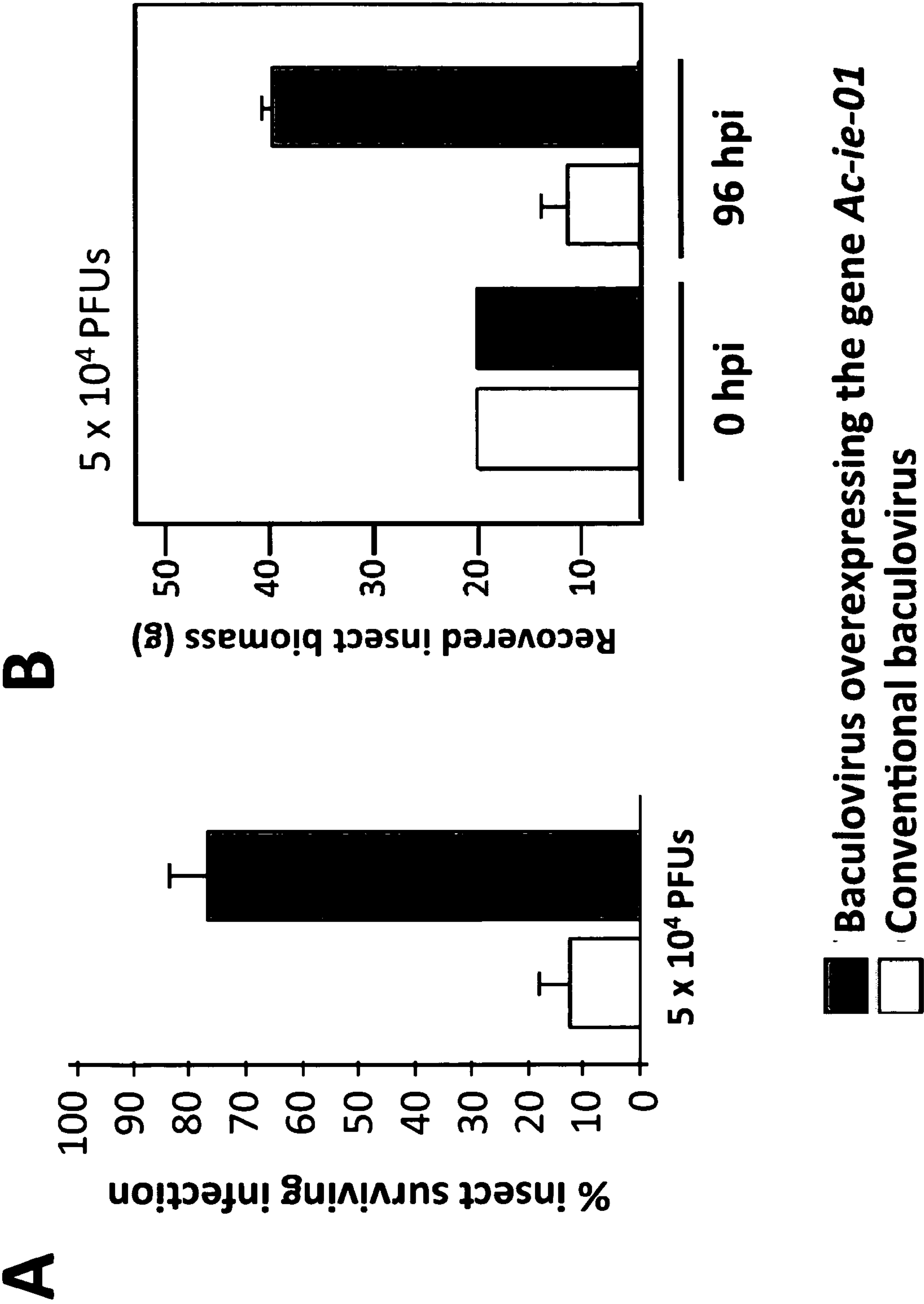
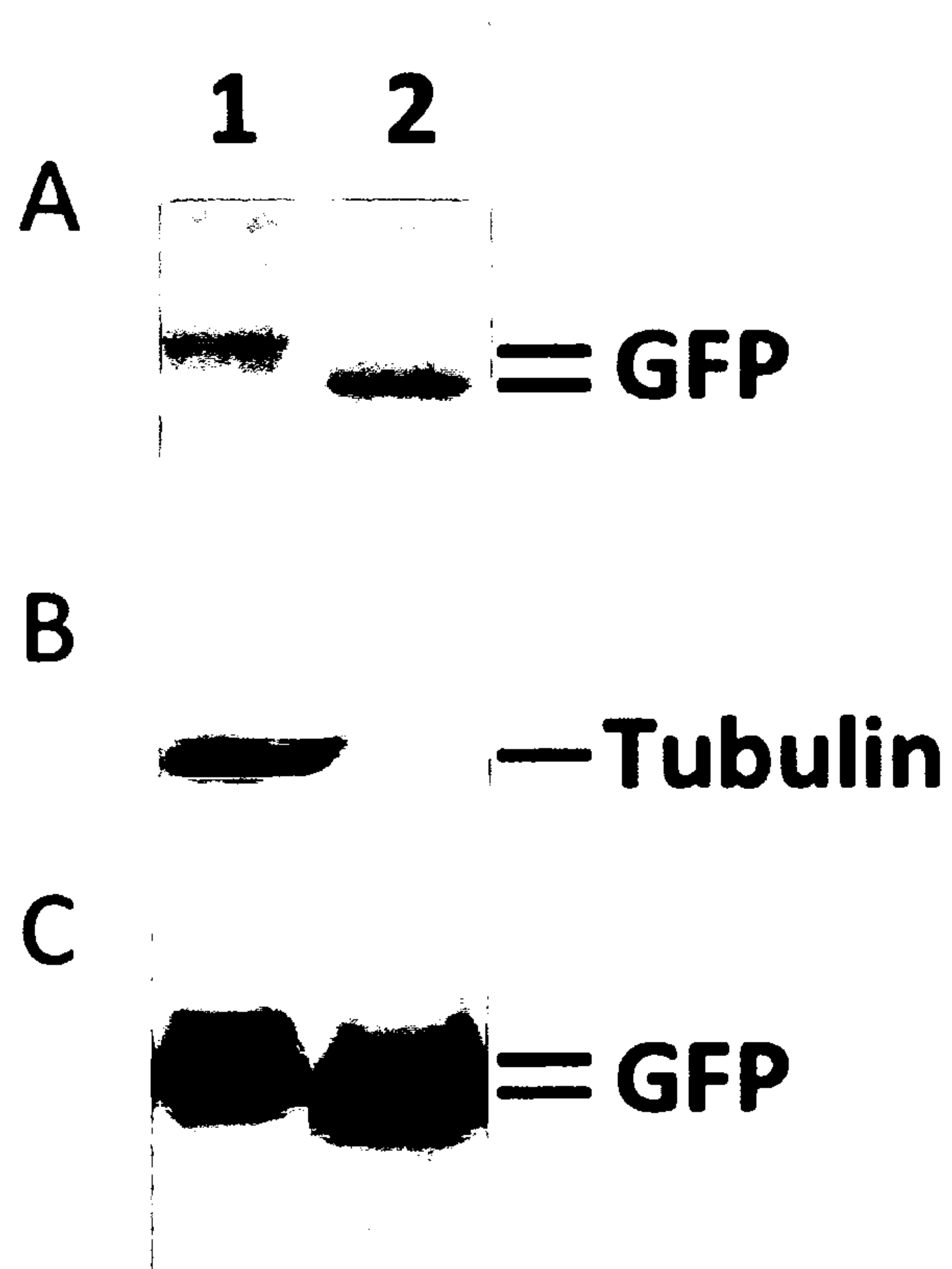


Figure 6

**Figure 7**

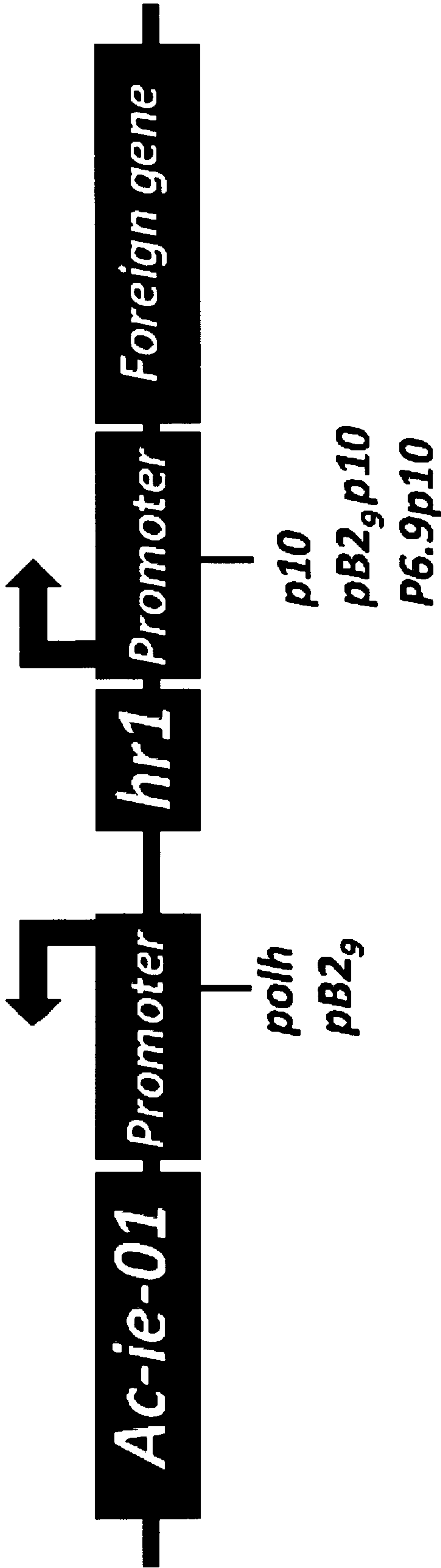


Figure 8