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(54) Title: METHOD AND SYSTEM FOR INDUCIBLE RECOMBINATIONAL CLONING IN BACTERIAL CELLS

(57) Abstract: The present invention discloses methods and systems that enable high throughput screening of genes and DNA sequence bearing expressed sequence tags in mammalian cells. The invention can easily be applied to the delivery of other functions like that of RNAi, ribozyme, antisense, DNA vaccine, etc.. The invention comprises a donor plasmid bearing a donor cassette and a receiver plasmid bearing a recombinase cassette, which may be propagated and maintained in bacterial culture. Through the use of inducible recombinational cloning, the donor cassette comprising a transgene sequence and a receiver plasmid, are easily and efficiently recombined *in vivo* to create a target vector comprising desired motifs (e.g. splice sites, introns and polyadenylation sequences). The present invention also provides convenient means for the introduction of the transgene into the donor cassette and provides an ability to create a library of transgene sequences for study maintained either in the donor plasmid or in the target vector. Because of the ease with which genes and putative genes may be introduced into the donor plasmid, and that recombination occurs *in vivo*, the invention allows for the functional study of genomics in model systems.

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METHOD AND SYSTEM FOR INDUCIBLE RECOMBINATIONAL CLONING IN BACTERIAL CELLS

Reference to Related Applications

This application claims priority to U.S. Provisional Patent Application No.
5 60/330,910 entitled "Method and System for Inducible Recombinant Cloning in
Bacterial Cells" filed November 2, 2001, which is hereby completely and entirely
incorporated by reference.

Background of the Invention

1. Field of the Invention

10 In general, the field of the present invention is a method and system for the
high throughput screening of genes or DNA sequences with expressed sequence tags
using *in vivo* recombinational cloning. Sequences cloned are inserted into shuttle
vectors by the *in vivo* induction of recombinase creating a target vector optimized
for mammalian expression.

15 2. Description of the Background

The revolution in biotechnology resulting from new techniques in molecular
biology has led to new, and previously impractical, ways to pursue the investigation
of Genomics. Genomics is the study of the DNA sequences, the identification of
genes and the analysis of the physiological function of those genes. The knowledge
20 derived from genomics will allow for an understanding of the molecular processes
that underlie life and also revolutionize agriculture, medicine, and many aspects of
healthcare. Since 1990, human gene discovery and functional genome studies have
been dramatically enhanced by the Human Genome Project, and by tremendous
effort from the entire scientific community and biomedical industry. As the Human
25 Genome Project nears completion and the sequences of over 30,000 genes are
identified the next goal is to identify their function and analyze their effects *in vivo*.

Efficient interpretation of the functional properties of human genes and other DNA sequences requires that resources and strategies be developed to enable large-scale investigations across whole genomes. A technically challenging first priority is to generate complete sets of full-length cDNA clones and sequences for human
5 and model-organism genes. Other functional-genomics goals include, studies into gene expression and control, creation of mutations that cause loss or alteration of function in non-human organisms, and the development of experimental and computational methods for protein analyses.

With the sequencing of the human genome the number of full-length DNA
10 sequences already available for functional testing is in the tens of thousands. In addition, there are well over 50,000 sequences with expressed sequence tags (ESTs) identified. Current efforts for functional genomics are focused on: 1) use of the laboratory mouse for examining gene function, particularly for mouse genes similar to those found in human genomic regions; 2) creation of a large mouse-mutation
15 resource for function studies; 3) comparison of human chromosome sequence with a region of mouse chromosome containing similar genes; 4) application of phage display to select single-chain antibodies to each gene product; 5) determination of gene function by the use of ribozymes or RNAi technologies to selectively inactivate the expression of any target gene; and 6) identification of the function of
20 the multiple proteins raised from a single gene which result from alternative splicing and editing of the mRNA, post-translational protein modification, or alternative translation.

To accomplish the above and other functional genomic studies, a major challenge is the efficient introduction of large numbers of full-length cDNA open
25 reading frames (ORFs) and nucleotide sequences with expressed sequence tags into target cells and tissues. It is through *in vivo* study, and investigation that the physiological activities and the functional properties of genes and putative genes can be readily revealed and identified. Elucidation of each gene's function *in vitro* or *in vivo* requires either direct delivery of the recombined protein or transfection of the
30 corresponding gene with certain vectors. The delivery of full-length cDNA possesses significant advantages, not only for *in situ* gene expression but, due to the

very large number of genes to be studied, is cost effective. Another advantage of the use of full-length cDNA is that with it gene delivery can be adapted into a high-throughput process for the rapid investigation of the physiologic actions of gene products. A more efficient gene delivery technology is needed in the post-genome era and will be a powerful tool for functional genomic study. The fast growing
5 pools of full-length cDNA and nucleic acid sequences bearing expressed sequence tags require better and more sophisticated gene delivery vehicles.

Currently, generation of gene delivery vectors in a high-throughput (HTP) manner has proven very challenging. The traditional approaches for gene cloning
10 are mostly *in vitro* based. *In vitro* cloning may result in genetic rearrangements, deletions or insertions, occurring in the desired gene sequence. Moving target sequences into expression plasmids or shuttle plasmids, is often limited by the availability or suitability of restriction sites. Although the *in vitro* use of site-specific restriction endonucleases and ligases have been the most commonly used
15 methods, more and more recombinase-based cloning systems have demonstrated their unique advantages for large scale cloning strategies. Efforts to use DNA recombinase to improve the cloning efficiency of a specific target sequence have yielded three commercially available products: Life Technologies, Inc.'s Gateway cloning system (US Patent, 5,888,732 and 6,143,557, Hartley, et al. Nov.7, 2000);
20 Invitrogen's Echo system (US Patent, 5,851,808, Elledge, et al. Dec. 22, 1998) and Clontech's Creator system, all documents and materials of which are hereby specifically and entirely incorporated by reference. While these systems all take advantage of the Cre recombinase enzyme they require transfer of the cloned sequences to another vector for expression in model systems.

25 These products and systems use DNA recombinase to provide cloning vectors and are all *in vitro* based resulting in two concerns. First, *in vitro* systems create a loss of control, which adversely affects utility. Second, when *in vitro* recombined plasmids are subjected to transfection procedures, low efficiency of the transfection requires high copy numbers to be used which increases the probabilities
30 for rearrangements and compromise the integrity of the resulting clones. More importantly, all those systems are not specifically designed for the gene delivery

vectors. As a result, several critical components for gene expression in mammalian cells such as a splicing donor and receptor, and a polyadenylation signal are not present. Further, other methods, particularly those of Wilson *et al.* (U.S. Patent No. 6,274,354 hereby incorporated in its entirety) which take advantage of the cre-lox
5 system and are in vivo based are directed to methods for the production of vectors used in gene therapy.

Summary of the Invention

The invention provides methods and systems for the inducible recombination cloning of target sequences in bacterial cells, and also methods and
10 systems for the expression and analysis of transfection efficiency in mammalian host cells or organisms. In addition, the invention also provides an improved means of the subcloning of the target gene or PCR product into the vector greatly facilitating that procedure.

One embodiment of the invention is directed to a cloning method and system
15 wherein a transgene (e.g. a target gene or PCR product), is transferred into a donor vector via a topoisomerase cut-and-joining function, and is translocated into a receiver plasmid, through the action of recombinase, to create a target vector which is specifically designed to be an efficient mammalian expression vector.

The donor plasmid may contain a bacterial origin of replication and a
20 selectable marker comprising antibiotic resistance gene (ARG). In addition, the donor plasmid may contain a donor cassette that comprises a gene or nucleic acid sequence bearing an expressed sequence tag and further comprises a splicing acceptor site, a topoisomerase site and/or a multiple cloning site (MCS), a polyadenylation (poly A) site and a selectable marker conferring a different
25 antibiotic resistance from that found in the backbone of the donor plasmid. The 5' and 3' ends of the donor cassette are marked by recombinase recognition motifs. It is another aspect of the invention that the transgene or PCR product can be easily ligated into the donor plasmid at the site of the donor cassette, either by the use of topoisomerase or through the use of a conventional multiple cloning site (MCS).

Another embodiment of the invention that is directed to a receiver plasmid that contains a selectable marker in the backbone, that may be the same or different as that found in the backbone of the donor plasmid. In addition, the receiver plasmid contains a recombinase cassette which comprises recombinase recognition motif
5 compatible with the recombinase recognition motifs found in the donor plasmid. The recombinase cassette also contains a recombinase gene which is under the control of an inducible promoter such that induction of the gene results in the production of the recombinase enzyme. In addition, the recombinase cassette comprises an ARG, in the opposite orientation to the recombinase gene and different
10 from that found in the backbone of the receiver plasmid and donor plasmid or donor cassette. Further, 5' to the recombinase cassette is a viral promoter, expressed in eukaryotic cells, and an intron. 3' to the recombinase cassette is a further inducible promoter in the same orientation as the antibiotic resistance marker gene found in the recombinase cassette. Finally, there follows a further viral promoter which
15 drives a reporter gene.

By cotransfection of the host cells with the donor and receiver plasmids followed by induction of the recombinase gene in the recombinase cassette the donor cassette is translocated to the receiver plasmid. The recombinase cassette, lacking an origin of replication is not replicated in the bacterial cells and is, therefore
20 not propagated. The translocation of the donor cassette to the receiver plasmid creates a target vector or plasmid which now possesses a new combination of selectable markers derived from the backbone of the receiver plasmid and the donor cassette of the donor plasmid. The target vector can then be amplified in the bacterial host cells and is maintained through selection using the new combination
25 of antibiotic resistance that results from the translocation. Because of this selection process the target vector can be maintained in the host cells and a library of genes or PCR products may be maintained in bacterial cultures.

Because the target vector is designed to incorporate viral promoters, splice sites, intron sequences and mammalian expression sequences it is an efficient
30 expression vector for use to directly transfect mammalian cells or tissues. The arrangement of the splice donor, intron, recombinase recognition motif and splice

acceptor site allows those sequences to be spliced out upon transcription resulting in an mRNA sequence in which the viral promoter is directly followed the transgene of interest and a polyadenylation sequence allowing for extremely efficient translation of the target gene. This arrangement provides the means to study the function of the gene in mammalian cells, with very little manual manipulation of the DNA or vectors, by use of the host cells machinery to perform the molecular handling of the DNA and amplify the resulting plasmids.

Another embodiment of the invention is directed to bacterial cells that are transfected with a transformer plasmid comprising a bacterial origin of replication, an antibiotic resistance marker gene and an inducible promoter followed by a recombinase gene. In this embodiment, the donor plasmid is the same as described previously while, the receiver plasmid contains a recombinase cassette but lacks the recombinase gene. Upon induction of the recombinase gene the donor cassette is translocated to the receiver plasmid, conferring a new combination of antibiotic resistance marker genes.

Other embodiments and advantages of the invention are set forth, in part, in the following description and, in part, may be obvious from this description, or may be learned from the practice of the invention.

Description of the Figures

- Figure 1. Schematic Diagram of the Donor Plasmid.
- Figure 2. Schematic Diagram of the Receiver Plasmid.
- Figure 3. Bacteria are permanently transfected with the recombinase gene.
- Figure 4. A schematic diagram representing the cotransfection of bacteria with the Donor and Receiver plasmids.
- Figure 5. The recombination that occurs in bacterial cells.
- Figure 6. A schematic diagram illustrating the cotransfection of the Donor Plasmid and the Receiver Plasmid of Embodiment II.

- Figure 7. The diagram illustrates the results of the translocation that occurs when embodiment II of the invention.
- Figure 8. Illustrates the *in vivo* generation of shuttle and expression vectors using various viral promoters.
- 5 Figure 9. Illustrates the insertion of the donor plasmid into the different forms of the receiver plasmid shown in figure 8.
- Figure 10. Illustrates the stable transfection of a mammalian 293 cell with a target vector having an adenoviral promoter.
- 10 Figure 11. This figure illustrates that upon transfection of mammalian cells with the target vector, the post-transcriptional process results in the splicing out of the intron, LoxP splice acceptor sequence, resulting in an mRNA product.
- Figure 12. The expression of IPTG-inducible Cre from the Cre-expressing plasmid.
- 15 Figure 13. Example of successful transfer of donor cassettes into the receiver plasmids through co-transfection of the cre-expression bacterium.
- Figure 14. Another example of successful transfer of more donor cassettes into the receiver plasmids.

Description of the Invention

- 20 The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides new and improved methods for the study of genomics by allowing the efficient interpretation of the functional properties of human genes. The method provides surprisingly more efficient mechanisms to generate complete sets of full-length cDNA clones and sequences for
- 25 human and model-organism genes. In addition, the disclosed method allows a greater ability to study gene expression and control and creation of mutations that cause loss of function in non-human organisms.

The invention also provides new methods for the *in vivo* inducible recombinational cloning in bacterial host cells. In particular, the invention allows the recombination of a donor cassette found in a donor plasmid to a receiver plasmid by site specific translocation *in vivo* such that the subcloning from a shuttle vector, appropriate for bacterial amplification, to an expression vector appropriate for eukaryotic expression is carried out completely within the host cell. The resulting target vector is optimized to provide important sequences to facilitate the transcription of the transgene contained in the donor cassette. In addition, the instant invention also allows for ease of cloning of the gene or PCR product directly into the donor plasmid through the use of a topoisomerase action motif.

A "lox site" refers to a nucleotide sequence at which the product of the cre gene of bacteriophage P1, Cre recombinase, can catalyze a site-specific recombination. A variety of lox sites are known to the art including the naturally occurring loxP (the sequence found in the P1 genome), loxB, loxL and loxR (these are found in the E. coli chromosome) as well as a number of mutant or variant lox sites such as loxP511, lox.DELTA.86, lox.DELTA.117, loxC2, loxP2, loxP3 and loxP23. A recombinase refers to enzymes that recognize and bind to a short nucleic acid site or sequence and catalyze the recombination of nucleic acid in relation to these sites.

A "recombinase recognition motif" refers to a short nucleic acid site or sequence which is recognized by recombinase and which become the crossover regions during the site-specific recombination event. Examples of recombinase recognition motifs include, but are not limited to, lox sites, frt sites, att sites and dif sites. The term "frt site" refers to a nucleotide sequence at which the product of the FLP gene of the yeast 2 .mu.m plasmid, FLP recombinase, can catalyze a site-specific recombination.

It has been demonstrated that Cre-LoxP recombination *in vivo* is mechanistically different and much more tightly controlled than it is *in vitro*. Therefore, the present invention provides methods and a system for the inducible recombinational cloning of a donor cassette, bearing a transgene for functional

genomic study, with a receiver plasmid using site specific DNA recombinase (Cre) in bacterial cells and includes construction of several key components into the plasmids to create efficient mammalian expression vectors. The methods described in the present invention are specifically useful for HTP generation of expression
5 vectors (viral or non-viral) in mammalian cell systems and, as a result, provide a significant improvement over current technologies.

Molecular biology frequently makes use of "expression vectors" which refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably
10 linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, Kozak sequences, and termination and polyadenylation signals. By use of these genetic components
15 foreign DNA may be more efficiently expressed in model systems.

The terms "transformation" and "transfection" refer to the introduction of foreign DNA into prokaryotic or eukaryotic cells. Transformation of prokaryotic cells may be accomplished by a variety of means known to the art including the treatment of host cells with CaCl_2 , and electroporation, etc. Transfection of
20 eukaryotic cells may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

A donor plasmid denotes a series of nucleotide sequences bearing a bacterial
25 origin of replication and a selectable marker such that it can be specifically propagated *in vivo*. The term donor cassette denotes a series of nucleotide sequences flanked by recombinase recognition motifs which are capable of being translocated in a site specific manner from the site of one recognition motif to another.

In one embodiment, the invention provides a donor plasmid that comprises a donor cassette comprising a transgene for study and flanked by recombinase recognition motifs or *lox* sites. The invention also comprises a receiver plasmid that contains a transgene cassette, which comprises a recombinase gene under the control of an inducible promoter and is also flanked by *lox* sites compatible with those of the donor cassette. Upon induction of the promoter, which drives the recombinase gene the donor cassette is translocated to the receiver plasmid, creating a target plasmid suitable for expression in mammalian cells.

In another embodiment of the donor cassette, the cassette includes sequences important for the optimum translation in mammalian cells. For example, the presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40. In addition, these sequences may include but are not limited to Kozak sequences and polyadenylation sequences.

The Kozak sequence is based on upon the published consensus sequence for the translation start site consensus sequence of mRNAs, in some instances the Kozak consensus sequence is (G/A)NNATGG, (Kozak, 1991, Jour. Cell Biology, Vol. 115, pp. 887-903) (hereby incorporated in its entirety). The polyadenylation site denotes a DNA sequence, which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. Further, the cassette has a selectable marker comprising an antibiotic resistance marker gene such that the donor cassette can be specifically selected for. In yet another aspect of

the donor cassette it comprises a Topoisomerase I Action Motif and/or a multiple cloning site so that the transgene of interest can be ligated directly into the donor cassette within the donor plasmid.

5 The backbone of the donor plasmid also contains a bacterial origin of replication and second selectable marker which is different from the selectable marker found within the donor cassette. This combination allows the plasmid to be propagated in bacteria and ensures that the plasmid is maintained in *E. coli* or another appropriate bacterial strain with the due to the selectable marker found in the donor cassette. It is a further aspect of the invention that the target transgene or PCR
10 product may be cloned into the donor plasmid and the plasmid with transgene insert may be maintained in bacterial culture such that a library of cloned donor plasmids may be created and maintained awaiting recombination with the receiver plasmid.

The invention also comprises a receiver plasmid that also includes a selectable marker and an origin of replication such that the receiver plasmid may be
15 maintained in bacterial cells. In addition, the receiver plasmid also contains a transgene cassette. The recombinase cassette comprises an inducible promoter which drives a recombinase gene which may be the Cre recombinase or the FLP recombinase gene flanked by recombination recognition motifs. A recombinase is an enzyme that recognizes and binds to a short nucleic acid site or sequence and
20 catalyzes the recombination of nucleic acid in relation to these sites. Cre is a 38-kDa site-specific DNA recombinase from bacteriophage P1 that mediates recombination between DNA sequences at specific loci, called *lox* sites. These sites comprise two 13-bp inverted repeats separated by an 8-bp spacer region that provides directionality to the recombination reaction; the fixed directionality of this
25 8-bp region keeps the orientation and reading frame of the gene of interest intact following transfer to another vector. In addition, the recombinase cassette includes another selectable marker gene in the opposite orientation, which is different than that found in the donor cassette or the backbones of either the donor or receiver plasmid.

In another embodiment, the receiver plasmid contains a strong viral promoter and an intron sequence outside of the recombinase cassette on the 5' side. On the 3' side of the cassette is an inducible promoter in the same orientation as the antibiotic resistance marker gene found inside the cassette. 3' to the inducible promoter is
5 another viral promoter which drives a reporter gene. The viral promoters may be, but are not limited to, the cytomegalovirus promoter, the rous sarcoma virus promoter the lentivirus promoter, the herpes simplex virus promoter the adenovirus promoter or any other similar promoter. The inducible bacterial promoter may be, but is not limited to the T7/Lac, Tet, trc or Lac promoters. An inducible promoter is
10 a promoter that will turn on transcription of the gene it controls upon stimulation with a particular agent or condition such as change in temperature, addition of IPTG, and many other factors. For example, IPTG: Isopropyl-beta-D-thiogalactoside, induces the lac promoter. The architecture of the receiver plasmid allows the inducible bacterial promoter on the 3' side of the recombinase cassette to drive the
15 antibiotic resistance gene within the cassette only after recombinase mediated translocation has occurred. Further, the viral promoter drives the reporter gene only when the plasmid has been transfected to eukaryotic cells.

The invention thus allows for bacterial cells to be cotransfected with the donor plasmid and the receiver plasmid each bearing a selectable marker in the
20 plasmid backbone. In some instances the selectable markers in the backbones of the plasmids may be the same. It is also evident that the selectable markers may be different such that upon cotransfection the host cells will be resistant to two different antibiotics conferred by the selectable markers in the backbone of the plasmids. Induction of the recombinase gene initiates the translocation of the donor cassette
25 into the receiver plasmid at the site occupied by the recombinase cassette, this construct now forms the target plasmid. The recombinase cassette is removed and since it lacks a bacterial origin of replication it is not propagated in the host cell. Because the donor cassette has a selectable marker different from that of the backbone selectable marker a new combination of selective pressure can be applied,
30 combining the antibiotic resistance encoded by the receiver with that encoded by the donor cassette. This allows only those products which have the correct

recombination between receiver plasmid and donor cassette to be selected for and propagated within the host cell.

In a further embodiment, the host cells may be stably transfected with a transformer plasmid which comprises an inducible promoter driving a recombinase gene followed by an antibiotic resistance marker gene in the opposite orientation
5 driven by an inducible *Lac* promoter. In this embodiment the receiver plasmid contains a recombinase cassette flanked by recombinase recognition motifs, as described above, but it lacks the recombinase gene. As described, the cells are cotransfected with the receiver plasmid and the donor plasmid and the induction of
10 the recombinase gene allows the translocation of the donor cassette to the receiver cassette creating the target plasmid and conferring a new combination of antibiotic resistance as previously described.

The formation of the target vector brings the active sequences of the donor cassette into functional alignment with the genes that previously flanked the
15 recombinase cassette of the receiver plasmid. The target vector represents a combination of genes thus creating a mammalian expression vector. This vector comprises in 5' to 3' order a viral promoter, suitable for strong constitutive expression in mammalian cells, such as the cytomegalovirus promoter, an intron sequence, a recombinase recognition motif, a splicing acceptor site, a possible
20 Kozak sequence, and the transgene of investigation, a polyadenylation sequence, all of these will facilitate gene expression in mammalian cells. The resulting vector also possesses the antibiotic resistance gene that is in the opposite (3'-5') orientation from the rest of the nucleotide sequences received from the donor plasmid. In addition, these sequences are followed by the second recombinase recognition motif,
25 the latter is followed by a second viral promoter such as the rous sarcoma virus promoter. The viral promoter drives a reporter gene which in some cases may be the green fluorescent protein or any other gene product that according to the researcher's choice. Because the reporter gene is driven by a viral promoter it is silent until the target plasmid is transfected into mammalian cells.

Once the target plasmid is created and selected for by the appropriate combination of antibiotics, the vector can then be purified from the *E. coli* and transformed into the model cells or organism of choice. Because of the unique architecture of the target plasmid resulting from the recombination of the donor and receiver plasmids the construct is uniquely suited for expression in mammalian cells. Upon transcription of the plasmid DNA the intron sequence will be spliced out between the splice donor site and the splice acceptor site. This process also removes the recombinase recognition motif situated between the two splice sites. An individual with ordinary skill in the art will realize that the invention herein described represents a significant advance over current recombination technology. In the present invention the recombination, cloning and selective steps all occur in vivo, hence there is no need for any of the apparatus currently necessary for in vitro cloning, including varying buffer solutions for each step gel purification and serial amplification of the target gene sequence. These steps all occur in the bacterial host cells. In addition, one with skill in the art will realize that the selectable markers may include antibiotic resistance genes or genes which encode an enzymatic activity that confers the ability to grow in medium lacking what would otherwise be an essential nutrient.

The following examples are offered to illustrate embodiments of the present invention, but should not be viewed as limiting the scope of the invention.

Examples

The donor plasmid shown in Figure 1 contains an origin of replication and an antibiotic resistance marker gene in the vector backbone. The donor cassette is illustrated flanked by recombinase recognition motifs. The cassette also contains multiple cloning sites and/or a Topoisomerase I Action Motif (TAM) for insertion of the transgene sequence. The optional Kozak sequence is also shown in the figure. Since the TAM has the 5'-CCCTT-3' Topo-I recognition sequence, it could be designed to enable the complementing DNA strain to probably position a nucleotide "G" in the "-3" position relative the the start codon ATG, creating a Kozak sequence which will facilitate the expression of interestd gene in eukaryotic cells. A second

antibiotic resistance gene without a promoter sequence is harbored downstream of the MCS in an opposite orientation. A non-translational DNA leader sequence is built in and located between left recombinae motif and the transgene insertion site. A splice acceptor sequence follows after the left recombinae motif. The Poly A
5 signal sequence follows the insertion site.

Two embodiments of the receiver plasmid are shown in Figure 2. The upper figure shows the presence of the inducible promoter followed by the recombinae gene; the lower figure lacks the promoter and gene. The receiver plasmid contains both an origin of replication and an ARG in the backbone. Both embodiments
10 contain a constitutively expressed reporter gene, which indicates transfection efficiency in mammalian cells.

Embodiment I: A cytomegalovirus (CMV) promoter drives transgene expression in mammalian cells. An intron precedes the recombinae cassette. The recombinae motif is followed by a T7/Lac promoter, which drives the recombinae
15 gene. A kanamycin resistance gene, in the opposite orientation, is followed by the second recombinae recognition motif. Outside of the right recombinae recognition motif is a second Lac promoter in the same orientation as the kanamycin gene driving expression of that ARG. A second viral promoter follows driving the expression of the reporter gene, in this instance the sequence for the green
20 fluorescent protein.

Embodiment II of the invention of the receiver plasmid lacks the promoter/recombinae sequences within the recombinae cassette and there is only one recombinae recognition motif since the design requires insertion of the transgene cassette not translocation of the recombinae gene.

25 Figure 3 shows bacteria permanently transfected with the recombinae gene when the receiver plasmid of Embodiment II.

Figure 4 depicts a schematic diagram representing the cotransfection of bacteria with the Donor and Receiver plasmids.

Figure 5 illustrates the recombination that occurs in bacterial cells. Upon induction of the recombinase gene the recombinase cassette is spliced out and the donor cassette is inserted. This recombination creates the target vector.

5 Donor Plasmid and the Receiver Plasmid of Embodiment II. This figure illustrates that when Embodiment II of the invention is used the plasmids are transfected into bacterial cells which already are permanently transfected with the recombinase transformation cassette.

10 Figure 7 shows a diagram illustrating the results of the translocation that occurs when embodiment II of the invention.

Figure 8 illustrates the *in vivo* generation of shuttle and expression vectors using various viral promoters. The receiver vector is designed in various forms to fit the purposes of the shuttle vectors for different expression vectors, such as naked plasmid vector and viral vector for direct gene delivery. The Lenti shuttle vector,
15 uses the Lenti virus promoter; HSV1, uses the Herpes Simplex I viral promoter; Av1 uses the adenovirus promoter and pCMV, uses the cytomegalovirus promoter.

Figure 9 illustrates the insertion of the donor plasmid into the different forms of the receiver plasmid shown in figure 8.

20 Figure 10 illustrates the stable transfection of a mammalian 293 cell with a target vector having an adenoviral promoter.

Figure 11 illustrates that upon transfection of mammalian cells with the target vector, the post-transcriptional process results in the splicing out of the intron, LoxP splice acceptor sequence, resulting in an mRNA product.

25 Figure 12 shows the expression of IPTG-inducible Cre from the Cre-expressing plasmid. The plasmid expressing IPTG-inducible Cre was constructed, named pIntQE60Cre. The figure shows the IPTG-induction of Cre in two of the pIntQE60-transformed M15(Rep4) bacteria colonies. A mini-induction was carried out, with 2 ml LB, 1 mM IPTG, 2 hours of induction at 37°C.

Table 1 provides an in vivo recombination. To test the idea of in vivo recombination within Cre-expressing bacteria, the commercially available donor plasmids (pDNR-1r and pDNA-LacZ) and receiver (pLPcmvNeo), all from Clontech, our home-made receivers (pIntCI.R1 and pIntCI.R3) and viral shuttle/receivers (pIntAV1-SpaT2 and pIntAv1-SpaT3) were tested in the co-transformation assay with the Cre-expression bacterium, IntCreQT. The table shows the co-transformation resulting Cm-resistant bacteria colonies. The successful gene transfer within these colonies was proved by enzymatic analysis.

Figure 13 shows an example of successful transfer of donor cassettes into the receiver plasmids through co-transfection of the cre-expression bacterium. SEAP/Cm (chloramphenicol resistance gene) and LacZ/Cm were transferred, respectively, from donor plasmid into the receiver plasmid, pint-Av1-SpaT. The resulting target plasmid DNAs were isolated from transformant bacteria, and analyzed by BglII/Xho restriction digestion. Lane 1: The receiver plasmid control, resulting in three DNA fragments (4.2, 2.3, and 0.2 kb). Lane 2 & 3: the receiver with SEAP/Cm insert; because of the insertion, the 4.2 Kb fragment changed to 6.6 Kb, as expected. Lane 4 & 5: the receiver with LacZ/Cm insert, the 4.2Kb fragment changed to 8.0 Kb.

Figure 14 shows another example of successful transfer of more donor cassettes into the receiver plasmids. Seventeen more cDNAs were transferred into receiver and analyzed with EcoRI digestion the similar way as described in Figure 13. Transfer of four representative genes are presented in this picture. Lane 10 & 11: the receiver alone (Lane 1-3, and 13, IFN- α gene-containing receiver. Lane 4-6: TNF- α . Lane 7-9: IFN- β . Lane 14-16: VEG-1. Lane 17-19: IL-10. Except lane 6, all the mentioned lanes have shown the reasonable two DNA fragments with reasonable size, indicating successful gene transfer. The upper band of 5.568Kb of the vector had gained extra size of these genes (TNF- α , 882bp; IFN- β , 858bp; VEG-1, 1046bp; and IL-10, 854bp).

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention

disclosed herein. All references cited herein for any reason, including all U.S. and foreign patents and patent applications, are specifically and entirely incorporated by reference. It is intended that the specification and examples be considered exemplary only, with the true scope and spirit of the invention indicated by the

5 following claims.

Claims:

1. A method for *in vivo* inducible recombinational cloning in a bacterial host cell comprising:
transfecting said bacterial host cell with:
 - 5 a donor plasmid that contains a donor cassette comprising a first pair of recombinase-recognition motifs flanking a transgene, a donor selectable marker, and a donor inducible promoter positioned to transcribe the donor selectable marker when induced; and
 - a receiver plasmid that contains a receiver cassette comprising
10 a second pair of recombinase-recognition motifs flanking a receiver selectable marker, and a receiver inducible promoter positioned to transcribe the receiver selectable marker when induced, wherein the second pair of recombinase-recognition motifs can functionally interact with said first pair of recombinase-recognition motifs during recombination; and
 - 15 inducing recombination of the transfected cell such that the transgene is translocated from said donor plasmid to said receiver plasmid creating a target plasmid wherein said transgene is capable of being subcloned into eukaryotic cells.
2. The method of claim 1 wherein the bacterial host cell is *E. coli*.
3. The method of claims 1-2 wherein the donor plasmid contains one or
20 more sites selected from the group consisting of a poly A site, a multiple cloning site, a splice site, a Kozak site, a topoisomerase site, and combinations thereof.
4. The method of claims 1-3 wherein the receiver plasmid contains one or more sites selected from the group consisting of a poly A site, a multiple cloning site, a splice site, a Kozak site, a topoisomerase site, and combinations thereof.
- 25 5. The method of claim 4 wherein the polyadenylation site is selected from the group consisting of a bovine growth hormone polyadenylation sequence, a simian virus 40 polyadenylation sequence, a herpes simplex virus thymidine kinase polyadenylation sequence, and combinations thereof.

6. The method of claim 4 wherein the topoisomerase site is a Topoisomerase I Action Motif.

7. The method of claims 1-6 wherein the first or second recombination recognition motifs are selected from the group consisting of loxP, loxP2, loxP3,
5 loxP23, loxP511, loxB, loxC2, loxL, loxR, lox.DELTA.86, lox.DELTA.117, ftr, dif, att and combinations thereof.

8. The method of claims 1-7 wherein the transgene is selected from the group consisting of cloned DNA sequences, PCR-amplified sequences, cDNA fragments, and combinations thereof.

10 9. The method of claims 1-8 wherein the selectable marker is selected from the group consisting of an antibiotic-resistance gene, a strA gene, a sacB gene, and combinations thereof.

10. The method of claim 9 wherein the antibiotic-resistance gene is selected from the group consisting of kanamycin resistance genes, ampicillin
15 resistance genes, tetracycline resistance genes, chloramphenicol resistance genes, streptomycin resistance genes, and combinations thereof.

11. The method of claims 1-10 wherein the donor plasmid contains two different donor selectable markers, one located within the donor cassette and the other located outside of the donor cassette.

20 12. The method of claims 1-11 wherein the receiver plasmid contains two different receiver selectable markers, one located within the receiver cassette and the other located outside of the receiver cassette.

13. The method of claims 1-12 wherein the donor plasmid contains a promoter positioned to transcribe the donor cassette.

25 14. The method of claims 1-13 wherein the receiver plasmid contains a promoter positioned to transcribe the receiver cassette.

15. The method of claims 13-14 wherein the promoter is selected from the group consisting of viral promoters, promoters suitable for expression in eukaryotic cells, and combinations thereof.

16. The method of claim 15 wherein the viral promoter is selected from the group consisting of cytomegalovirus promoters, rous sarcoma virus promoters, lentivirus promoters, vaccinia virus promoters, herpes virus promoters, adenovirus promoters, and combinations thereof.

17. The method of claims 1-16 wherein the receiver plasmid contains a reporter gene and a promoter suitable for expression of the reporter gene in eukaryotic cells which, upon recombination with the donor plasmid, becomes a part of the target plasmid.

18. The method of claim 17 wherein the reporter gene is a green fluorescent protein gene.

19. The method of claims 17-18 wherein expression of the reporter gene is an indicator of recombination efficiency.

20. The method of claims 1-19 wherein the receiver plasmid contains a second inducible promoter and a recombinase gene wherein the recombinase gene is positioned to be transcribed from said second inducible promoter when induced.

21. The method of claims 1-20 further comprising a transformer plasmid that contains a transformer inducible promoter and a recombinase gene wherein the recombinase gene is positioned to be transcribed from said transformer inducible promoter when induced.

22. The method of claims 20-21 wherein the recombinase gene is selected from the group consisting of Cre, Flp, and combinations thereof.

23. The method of claims 1-22 wherein the donor inducible promoter is oriented to transcribe the donor selectable marker when induced and that orientation is opposite the orientation of the transgene.

24. The method of claims 1-23 wherein the receiver inducible promoter is oriented to transcribe the receiver selectable marker when induced.

25. The method of claims 1-24 wherein the inducible promoters are selected from the group consisting of lac promoters, lac/T7 promoters, T7
5 promoters, Tet promoters, trc promoters, and combinations thereof.

26. The method of claims 1-25 wherein the target plasmid contains the donor selectable marker, the receiver selectable marker, or both.

27. The method of claims 1-26 wherein transcription of the transgene of said target plasmid is facilitated by the presence of, in 5' to 3' order:
10 a viral promoter;
a splice donor site;
an intron;
recombinase-recognition motifs flanking a splice acceptor site such that post-transcriptional processing of the transgene results in loss of a splice donor
15 site and an intron; and
a splice acceptor site.

28. A system for the inducible recombinational cloning in a bacterial host cell comprising:

a donor plasmid comprising a bacterial origin of replication, a first
20 selectable marker, a donor cassette comprising a transgene sequence flanked by a first set of recombinase-recognition motifs wherein 5' to the transgene sequence is a splice-acceptor site followed by a Kozak sequence, and 3' to the transgene sequence is a polyadenylation sequence followed by a second selectable marker;

a receiver plasmid comprising: a bacterial origin of replication, a
25 selectable marker, a recombinase cassette containing, in 5' to 3' order

a second set of recombinase-recognition motifs flanking a first inducible bacterial promoter

a recombinase gene; and a third selectable marker positioned in opposite orientation to the recombinase gene, wherein said first and second sets of

recombinase recognition motifs functionally interact;

a viral promoter 5' to said recombinase cassette suitable for expression in eukaryotic cells; and

an intron sequence; and

5 a second inducible bacterial promoter 3' to said recombinase cassette and positioned in the same orientation as the third selectable marker; and

a second viral promoter functionally linked to a reporter gene;

wherein induction of the second inducible promoter in the recombinase cassette allows for expression of the recombinase gene causing
10 translocation of the recombinase cassette from said receiver plasmid to said donor plasmid and translocation of said donor cassette to said receiver plasmid at the site occupied by said recombinase cassette, and formation of a target vector resulting from the translocation of said donor cassette to said receiver plasmid wherein said target vector is suitable for expression in eukaryotic cells.

15 29. The system of claim 28 wherein transfection of eukaryotic cells with said target vector results in expression of said recombinase gene from said viral promoter.

30. A system for the inducible recombinational cloning of a desired sequence in bacterial cells comprising:

20 a bacterial host cell stably transfected with a transformer plasmid comprising a first inducible bacterial promoter that promotes transcription of a recombinase gene positioned in one orientation, and a first selectable marker with a second inducible promoter positioned in the opposite orientation;

a donor plasmid comprising a second selectable marker, a donor
25 cassette that contains a transgene sequence flanked by a first set of recombinase recognition motifs wherein 5' to the transgene sequence is a splice acceptor site followed by a Kozak sequence, and 3' to the transgene sequence a polyadenylation site followed by a third selectable marker;

a receiver plasmid comprising a fourth selectable marker, a
30 recombinase cassette comprising, in 5' to 3' orientation, a second set of recombinase recognition motifs flanking a fifth selectable marker positioned in the opposite

orientation, and a second inducible bacterial promoter followed by a viral promoter functionally linked to a reporter gene, wherein said first set of recombinase recognition motifs functionally interact during recombination with said second set of recombinase recognition motifs, and wherein 5' to said recombinase cassette is a
5 viral promoter suitable for expression in mammalian cells followed by an intron sequence;

wherein induction of said recombinase gene allows for the translocation of said donor cassette to said receiver plasmid resulting in the creation of a target plasmid suitable for use as an expression vector in eukaryotic cells.

10 31. The system of claim 30 wherein transfection of eukaryotic cells with said target vector results in constitutive expression of said viral promoter.

32. The system of claims 30-31 wherein the first, second, third, fourth or fifth selectable marker is selected from the group consisting of kanamycin resistance genes, ampicillin resistance genes, tetracycline resistance genes, chloramphenicol
15 resistance genes, streptomycin resistance genes, strA genes, sacB genes, and combinations thereof.

33. The system of claims 30-32 wherein the first and second selectable markers are different.

34. The system of claims 30-32 wherein the third and fourth selectable
20 markers are different.

DONOR PLASMID AND FULL LENGTH cDNA

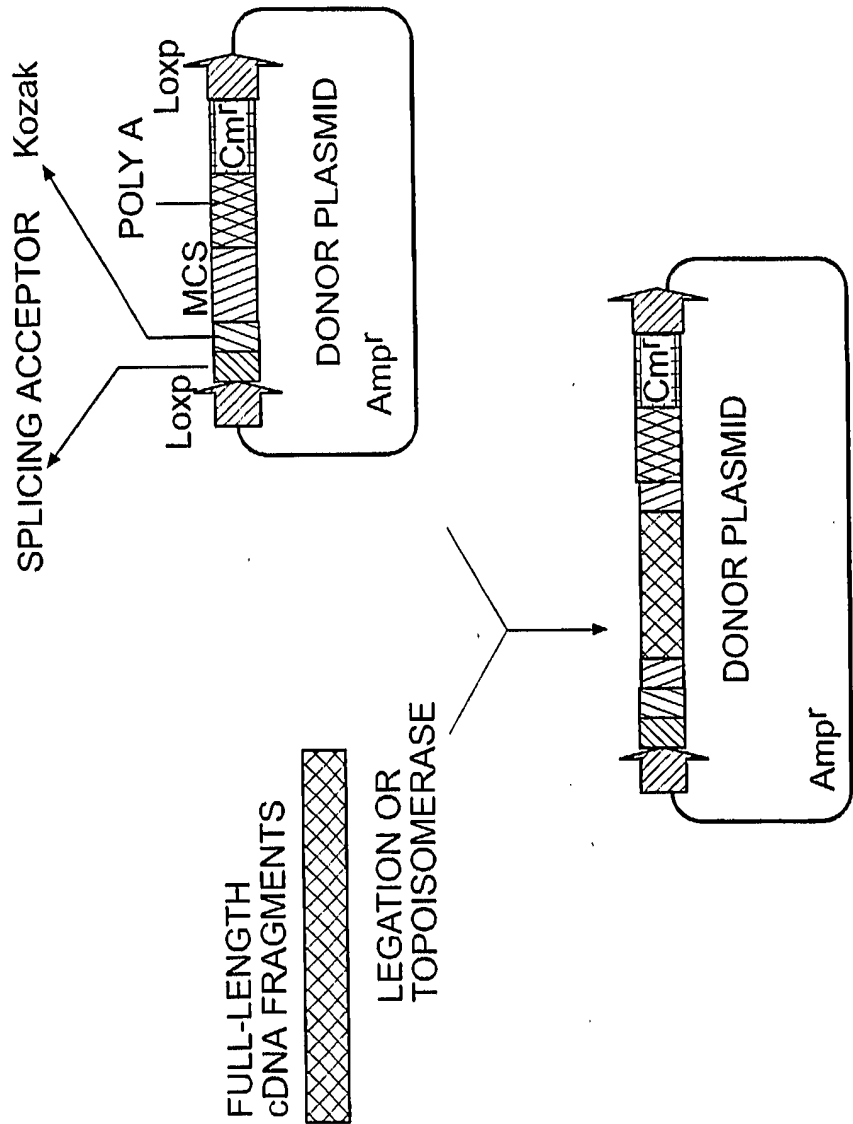


FIG. 1

RECEIVER PLASMID VECTORS

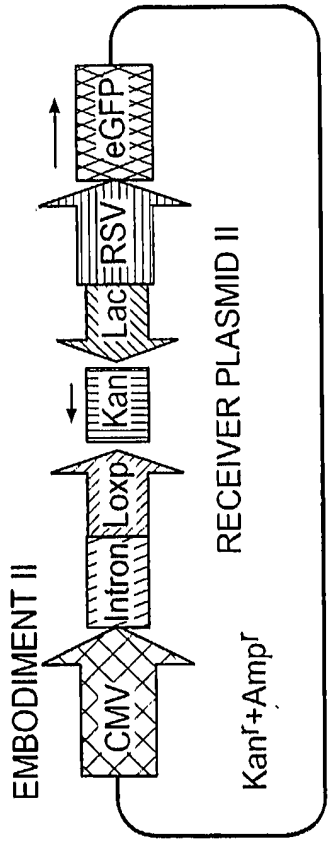
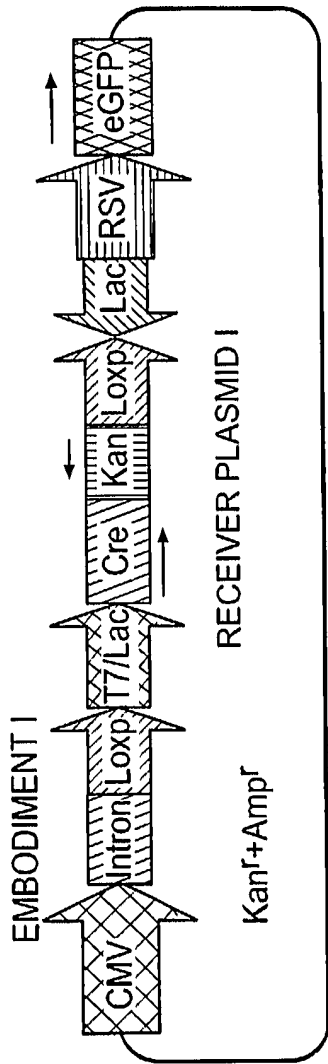


FIG. 2

RECOMBINASE EXPRESSION BACTERIA

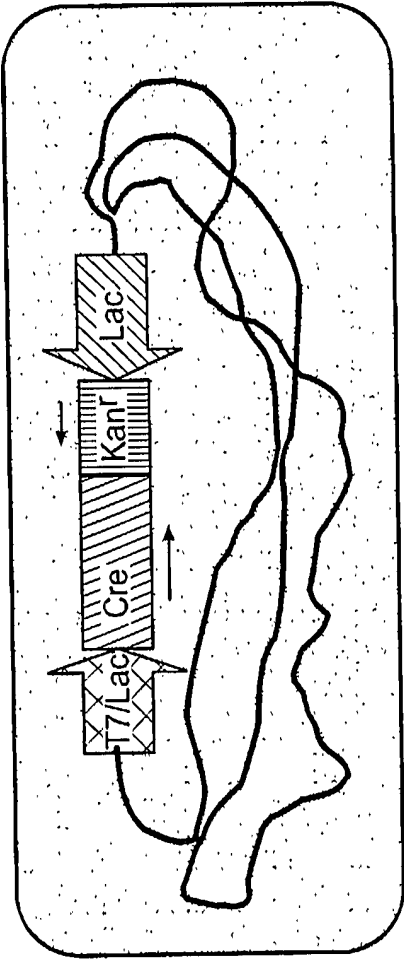


FIG. 3

CO-TRANSFECTION OF DONOR AND RECEIVER PLASMID I

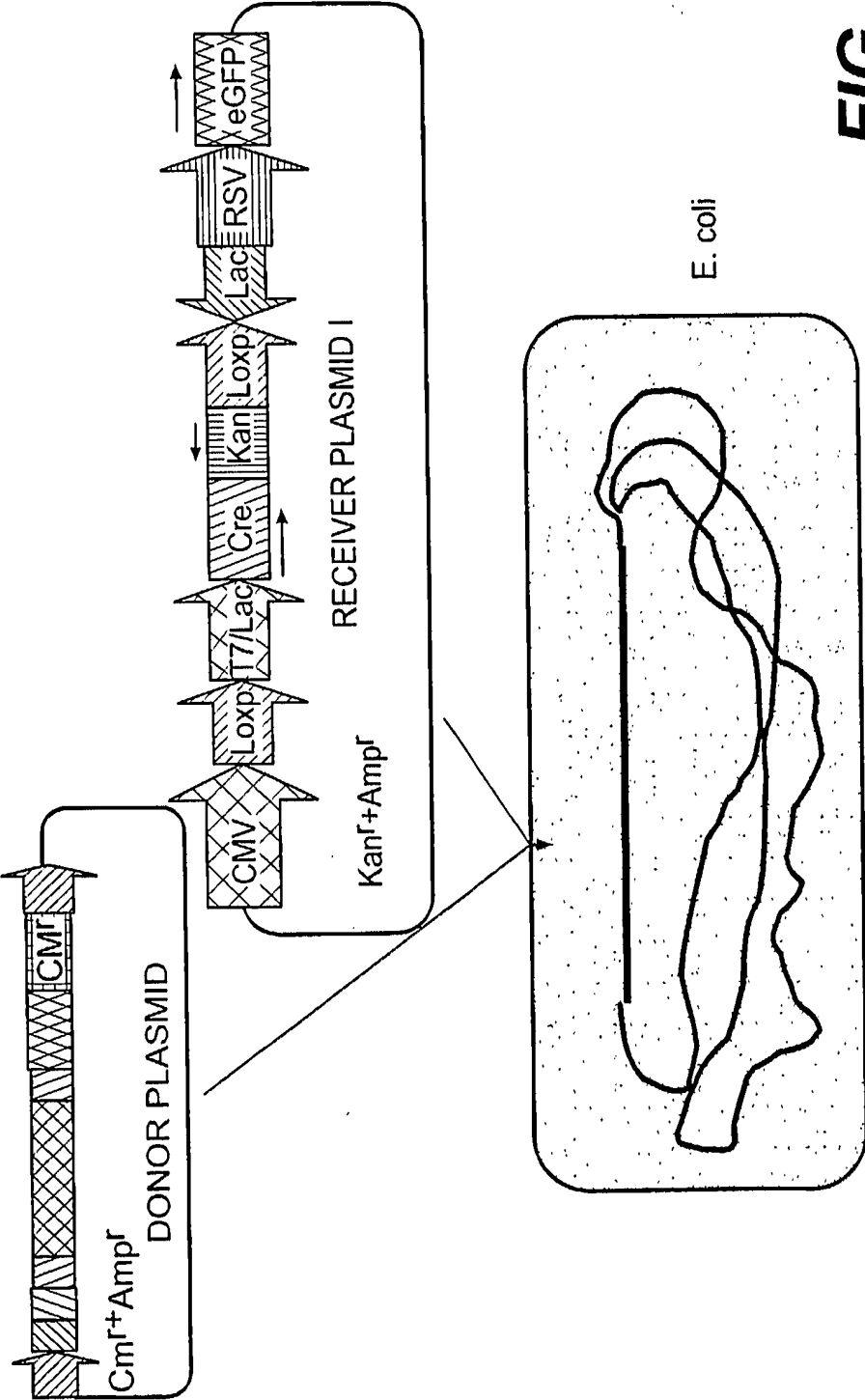


FIG. 4

RECOMBINATION IN *E. coli*, EMBODIMENT I

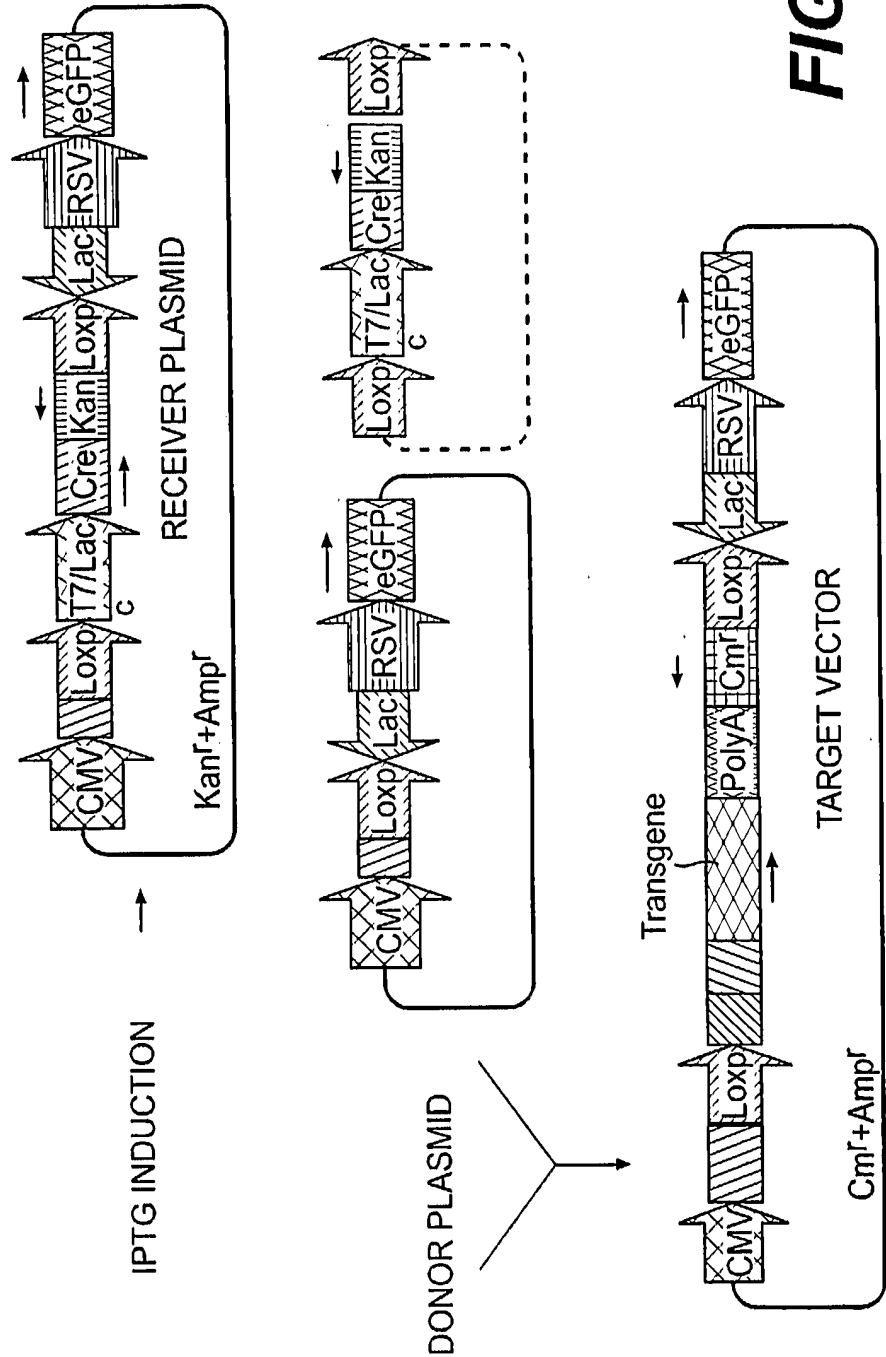


FIG. 5

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CO-TRANSFECTION OF DONOR AND RECEIVER PLASMID II

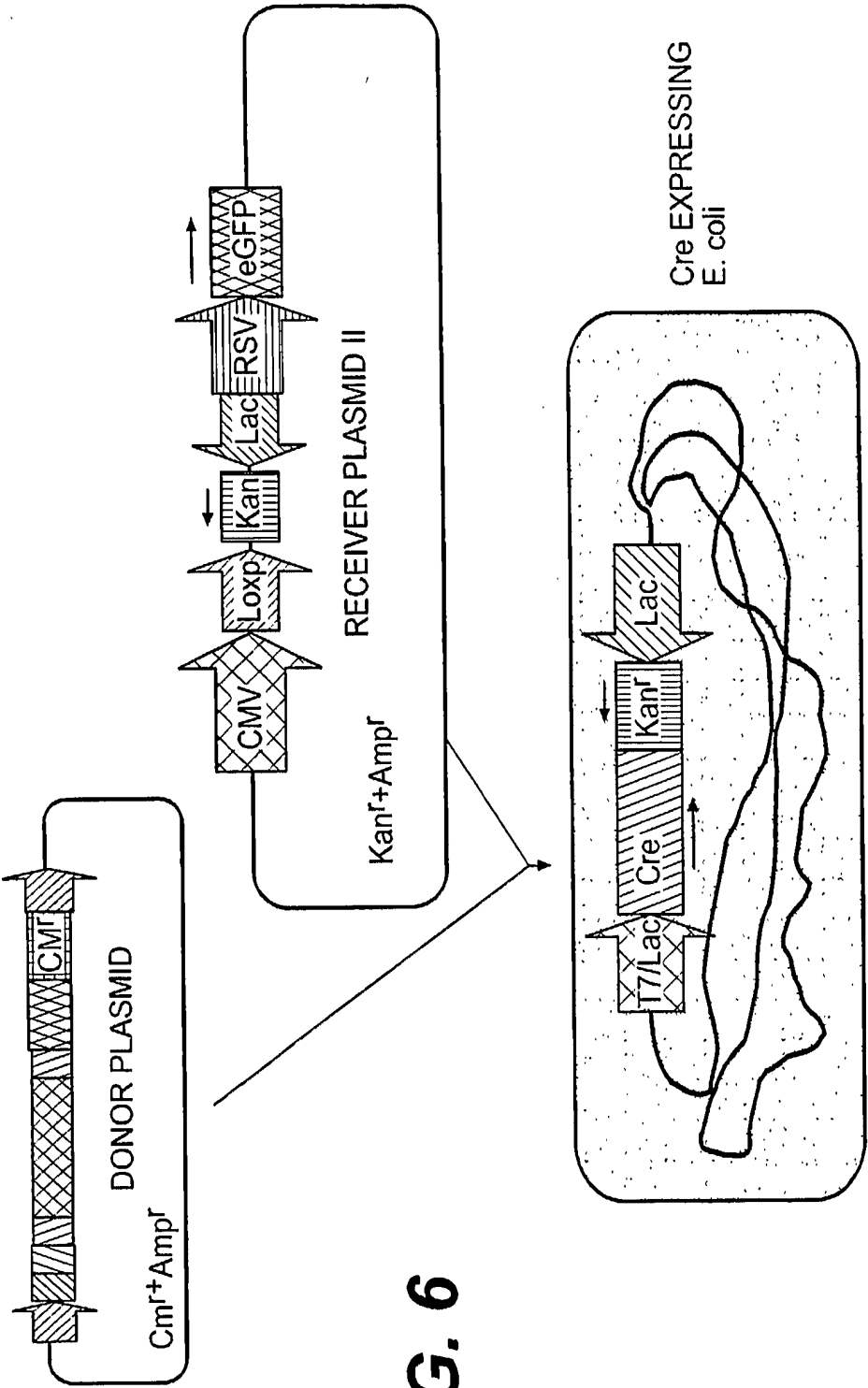


FIG. 6

RECOMBINATION IN E. Coli, EMBODIMENT II

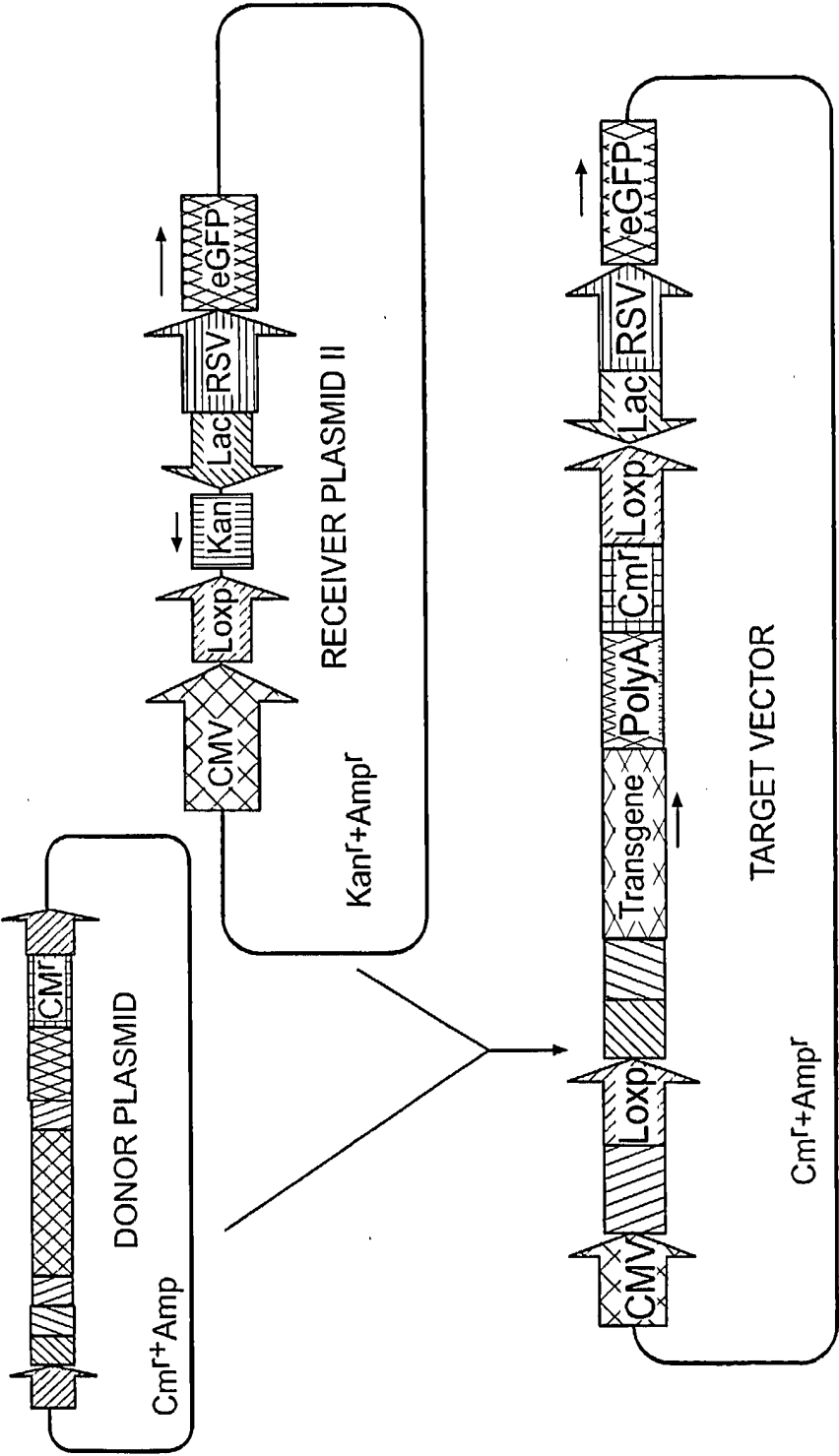


FIG. 7

IN VIVO GENERATION OF SHUTTLE AND EXPRESSION VECTORS

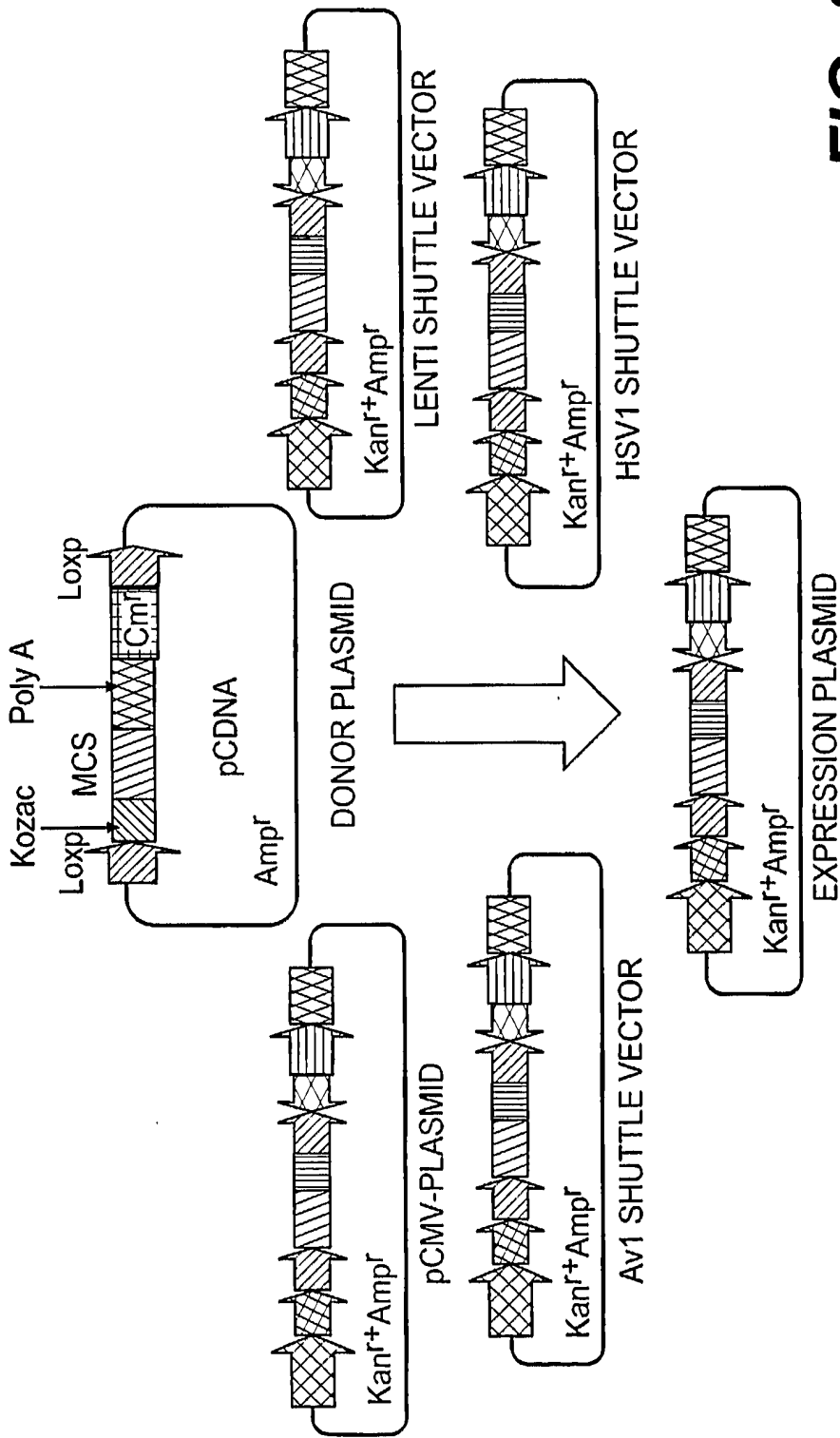


FIG. 8

HTP GENERATION OF SHUTTLE AND EXPRESSION PLASMID

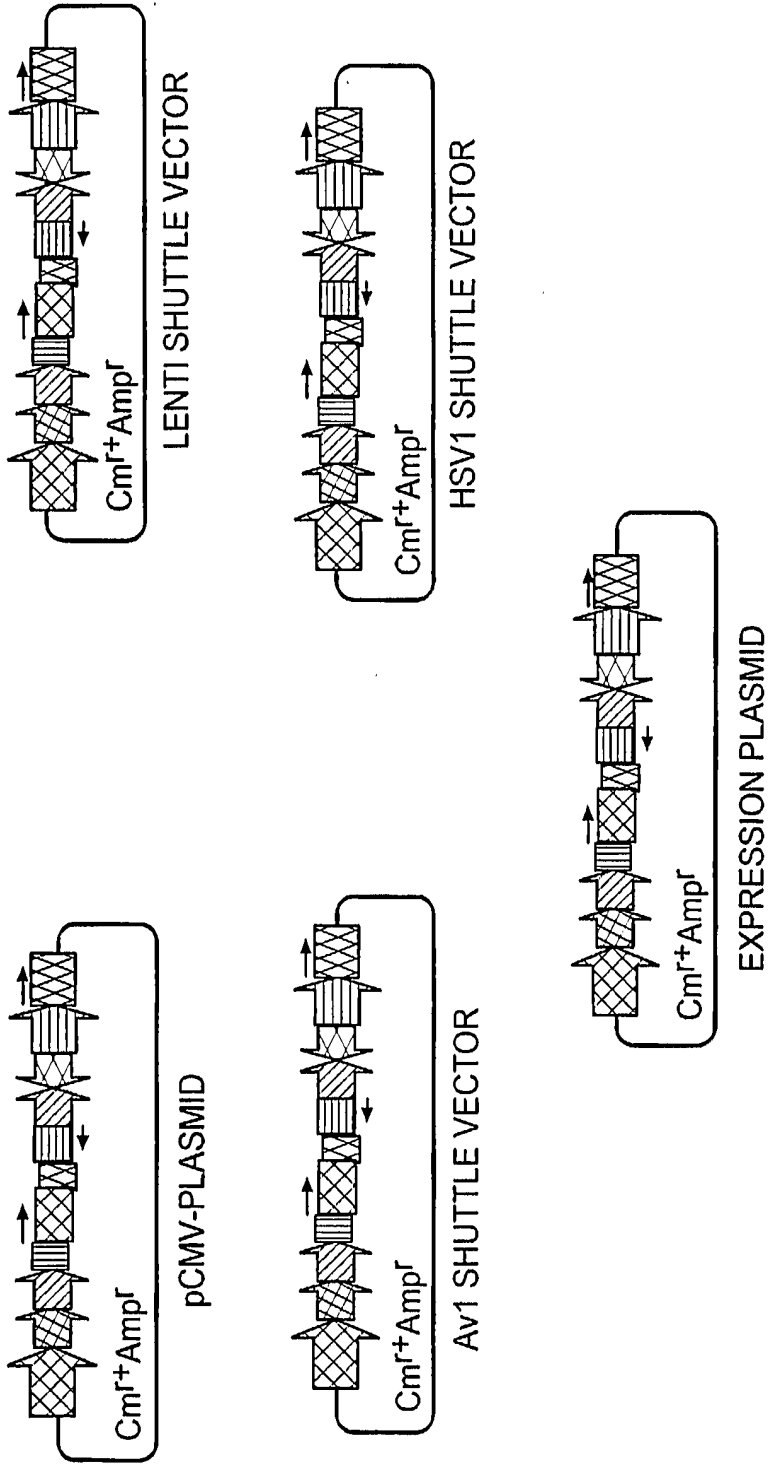


FIG. 9

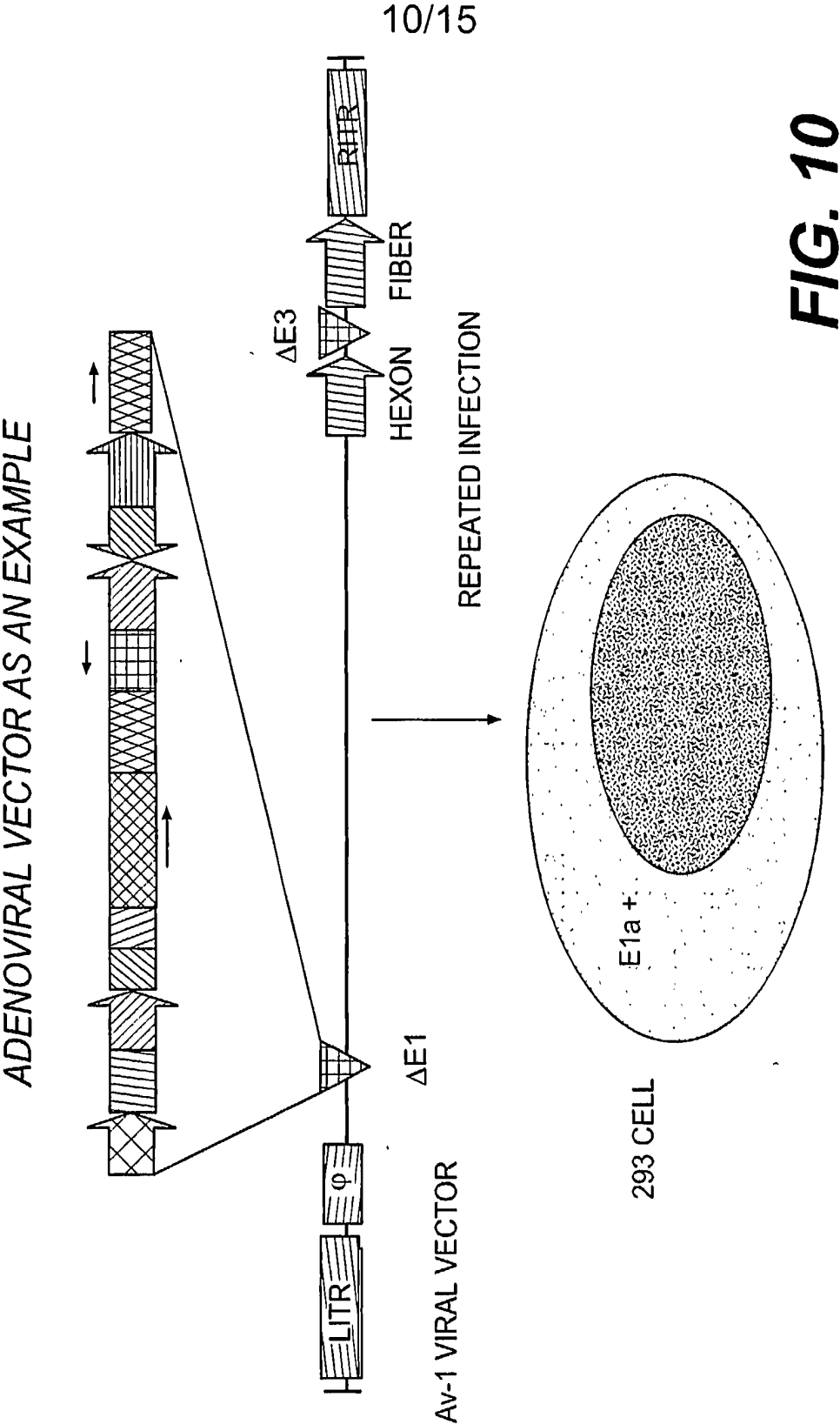


FIG. 10

REMOVE LoxP BY Intron SPLICING

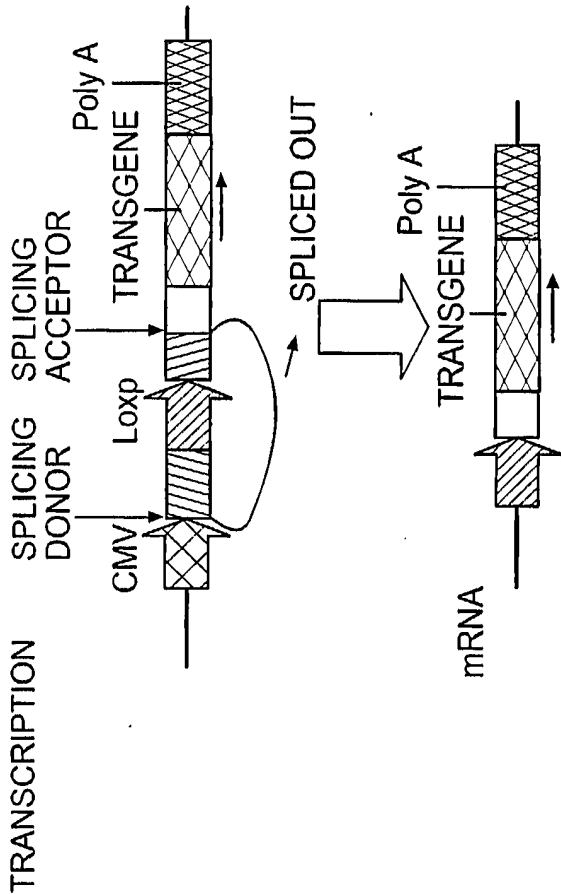


FIG. 11

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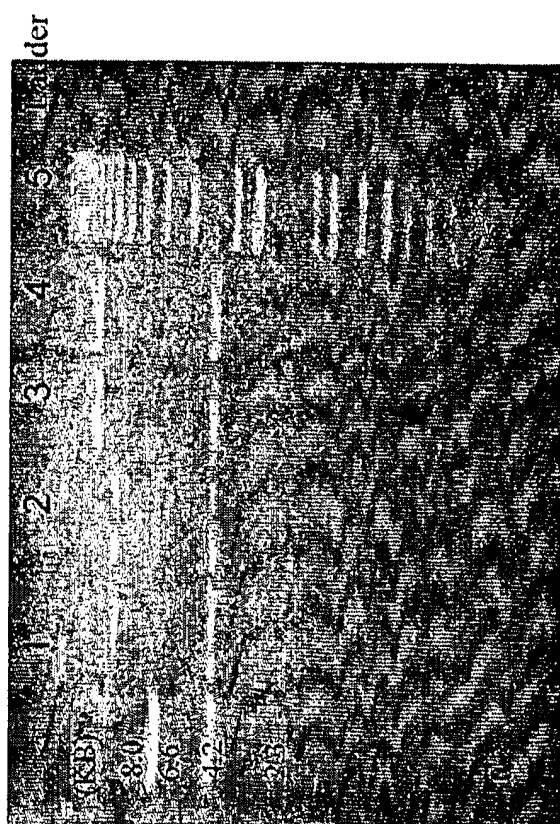


FIG. 12

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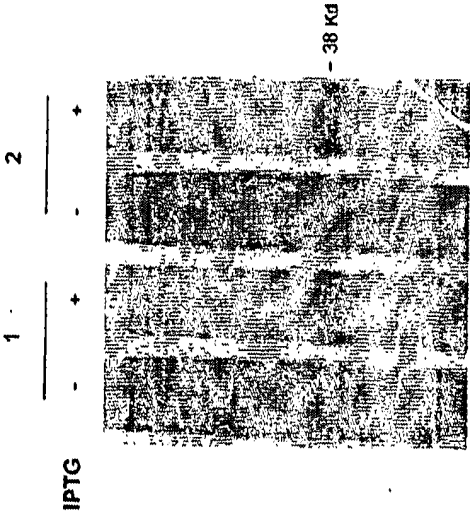


FIG. 13

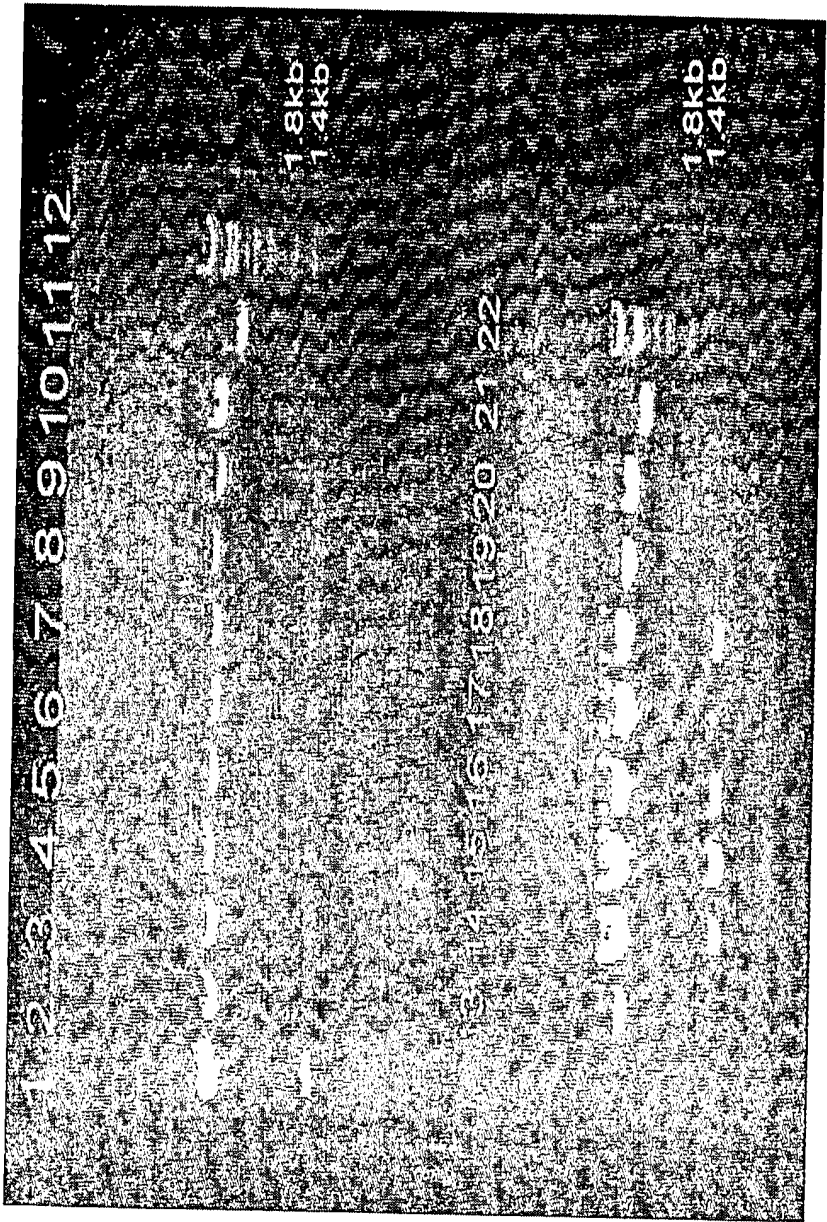


FIG. 14

Cre LoxP MEDIATED IN VIVO RECOMBINATION

PLASMID	1	2	3	4	5	6	7	8	9	10	11	12	13
pDNR-1r	100							100					
pLPcmvNeo		100						100	100				
pDNR-LacZ			100						100	100	100	100	100
pIntCI.R1				100						100			
pIntCI.R3					100						100		
pIntAV1SPaT2						100						100	
pIntAV1SPaT2							100						100
COUNTS/Cm ^r	0	0	0	0	0	0	0	1	6	3	4	43	26

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TABLE 1

FIG. 15