

# Integration of multiple expression cassettes into mammalian genomes in a single step

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## Method Article

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## Abstract

The modification of mammalian cells by the expression of multiple genes is a crucial technology in modern biological research. MultiLabel allows the modular assembly of independent expression units in a single plasmid which can be used for transient and stable modification of cells. In contrast to other methods, the assembly of the expression cassettes does not require restriction enzymes since it is recombination based. Here we describe strategies how genes of interest are efficiently cloned into MultiLabel vectors and how these expression vectors can be assembled by a Cre/LoxP reaction to a multigene plasmid. In addition, we describe how such plasmids are used to generate stable cell lines. Cloning and assembling of up to three plasmids is possible within two weeks, the insertion of a fourth and fifth expression cassette within another two weeks, and the generation of a stable cell line takes one to two months.

## Introduction

The simultaneous expression of multiple proteins in mammalian cells is an important technology in contemporary biological research. For instance, the coexpression of several fluorescently tagged sensors allows the concurrent observation of multiple parameters in living cells<sup>1</sup>. In addition, the simultaneous insertion of several genes is often necessary to change the fate of a cell e.g. when generating induced pluripotent stem cells<sup>2</sup>. Finally, the molecular and structural analysis of multiprotein complexes needs the heterologous expression of proteins in an appropriate host. Many strategies were developed in the last years to overcome the currently existing technical limitations. Cotransfection of independent expression constructs is the easiest strategy but it leads to a heterogeneous cell population with variable expression levels. It becomes especially inefficient if the cell line is difficult to transfect and if more than three proteins need to be expressed. Coinfection with numerous viruses may help, but the handling of viruses needs special safety requirements<sup>3</sup>. The different expression units can be assembled in a large plasmid with restriction enzymes but this strategy can not be standardized due to the limited number of restriction enzymes and the occasional occurrence of their recognition sites in genes of interest. IRES-based constructs are often used for the expression of two proteins<sup>4</sup>. Multiple proteins can also be expressed as a large precursor protein which is then processed by (self-) cleavage into its individual proteins. This strategy leads to proteins with modified N- and C-termini interfering sometimes with the functionality of the protein<sup>5</sup>. We previously used Cre/LoxP-mediated recombination to assemble multiple independent expression units in a single plasmid for production of protein complexes in Sf21 and E.coli cells for structural analyses<sup>6,7</sup>. Recombination-mediated assembly has the advantage that no restriction enzymes are necessary and that the expression vectors are therefore freely interchangeable. In addition, no special equipment laboratories are required. We recently developed a set of vectors, called MultiLabel, which allows the Cre/LoxP mediated multigene expression in mammalian cells<sup>8</sup>. It is possible to combine one acceptor vector with up to 4 donor vectors. These vectors have minimal backbone size to maximize transfection efficiency and they contain elements for the generation of stable cell lines (Supplementary Fig. 1). Here we describe the necessary steps to generate stable cell lines including cloning of the genes of interest in a MultiLabel vector backbone (Fig. 1A, steps 1 – 3), Cre/LoxP-mediated assembly of these vectors (Fig. 1B, steps 4 – 6), and transfection and selection of mammalian cell lines (Fig. 1C, steps 7 – 13). **Experimental Design** The choice of the appropriate acceptor vector is crucial for the successful application of our method. Ideally, the acceptor vector contains the gene of interest which is part of all planned multigene expression plasmids and which remains unmodified in all assemblies. The currently available acceptor vectors contain different resistance genes for the selection of stable cell lines. We recommend the use of pSI-AKR8 which contains a zeocin and a G418 resistance gene. These two genes are separated by a homing endonuclease site (I-SceI) which can be used for linearization prior to transfection (Figure 1C). Several acceptor vectors contain an FRT site from the Flp-In system of Invitrogen. The Flp-In system allows the Flippase-mediated integration of a plasmid at a defined site of the genome of a mammalian cell with an FRT site. We successfully integrated plasmids with a size up to 15 kb with this strategy. The choice of the donor vector is less crucial. These vectors are available with four different antibiotic resistance genes (ampicillin, spectinomycin, chloramphenicol, and gentamycin). A version with and without a cleavage site for the homing endonuclease I-CeuI exists for each antibiotic resistance gene. Additional expression cassettes can be inserted at this site by conventional cloning (see Supplementary Fig. S6 in Kriz et al. 2010 for details). Note that this site must not be used for the linearization step for the generation of stable cell lines because linearization at this point prevents expression of the upstream expression cassette. Classical cloning with restriction enzymes is described in the protocol but other methods such as SLIC or PCR-based methods can be used as well<sup>9,10</sup>. This is possible since not only the cloning sites are identical in all MultiLabel plasmids but also the adjacent 15 bp. The vectors do not contain a classical multi-cloning site. We used instead SapI (LguI) sites which allow seamless cloning (see Supplementary Figure S1 in Kriz et al. 2010 for details). This approach is not recommended if the first choice for transient transfections, but the plasmids

must be linearized for the generation of stable cell lines. We never obtained a cell line expressing four or five transgenes when using circular plasmids. The described method applies to the generation of stable porcine aortic endothelial cells (PAEC) and human embryonic kidney (HEK) cells. For other cell types, the method of transfection and the concentration of the selection antibiotics have to be adjusted. The text for the entire protocol can be found here: "Protocol in its entirety.doc":[http://www.nature.com/protocolexchange/system/uploads/1861/original/Exchange\\_protocol\\_berger.doc?1312282087](http://www.nature.com/protocolexchange/system/uploads/1861/original/Exchange_protocol_berger.doc?1312282087)

## Reagents

Cre recombinase and 10× reaction buffer (NEB) Phusion high-fidelity DNA polymerase in 2× HF or 2× GC buffer (Finnzymes) PCR primers (see "Supplementary Figure 2":[http://www.nature.com/protocolexchange/system/uploads/1866/original/Supplementary\\_Figure\\_2.pdf?1312282392](http://www.nature.com/protocolexchange/system/uploads/1866/original/Supplementary_Figure_2.pdf?1312282392) for details) Paraffin wax beads (e.g. Fluka Chemika, #411671) DpnI, AscI (Sgsl), SapI (Lgsl), and PacI (NEB, Fermentas) QIAprep Gel Extraction kit, QIAprep PCR purification kit (Qiagen) Spin Miniprep kit T4 DNA ligase, 10× T4 DNA ligase buffer (Fermentas) LB low-salt medium (Difco) DMEM or HAM-F12 serum free medium (Amimed) Fetal calf serum (Amimed) Penicillin-Streptomycin (Invitrogen) FuGENE HD (Roche) Opti-MEM I Reduced Serum Medium (Gibco) Template DNA (genomic, plasmid or cDNA) E. coli competent cells: TOP10, DH10β, BW23473 (pir+) MultiLabel vectors ("Supplementary Figure 1":[http://www.nature.com/protocolexchange/system/uploads/1865/original/Supplementary\\_Figure\\_1.pdf?1312282320](http://www.nature.com/protocolexchange/system/uploads/1865/original/Supplementary_Figure_1.pdf?1312282320)) Antibiotics (see Table 1) See figure in Figures section.

## Equipment

Thermocycler Equipment for agarose gel electrophoresis Electroporator and cuvettes Tissue culture Laboratory

## Procedure

**Construction of MultiLabel vectors with a single expression cassette** **1** This step can be performed using option A, B or C depending on the available template. Use option A, if a suitable expression cassette with a promoter, the gene of interest, and a polyA signal already exists. Standard primers for pcDNA (Invitrogen) or pEGFP type (Clontech) vector are described in this paper (Fig. 2A). Use option B, if a protein with an N-terminal fluorescent protein tag is needed (Fig. 2B). Use option C for the restriction enzyme free insertion of a coding region at any place in the MultiLabel vector (Fig. 2C). **A. First option:** This strategy inserts an existing expression cassette into MultiLabel vectors by conventional cloning. An existing expression cassette of a pcDNA (Invitrogen) or pEGFP-type (Clontech) vector is amplified by PCR using primers containing AscI and PacI restriction enzyme sites, and then cloned into a AscI- / PacI- cut MultiLabel vector (Fig. 2A). This strategy does not work, if your fragment contains an AscI or PacI site (in this situation ligate 3 fragments, use SLIC or a PCR-based method as described in option 1C). See the attached document for details on how to do this. "Procedure and Troubleshooting.doc":[http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure\\_and\\_Troubleshooting.doc?1312282214](http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure_and_Troubleshooting.doc?1312282214) **B. Second option:** This option describes the insertion of a gene of interest downstream of the coding region of a fluorescent protein by conventional cloning. MultiLabel vectors contain two SapI (Lgsl) restriction sites which generate non cohesive ends that can be used to exchange the linker fragment with your gene of interest (Fig. 2B). Make sure the SapI recognition sequence is not present in the genes you are cloning. See the attached document for details on how to do this. "Procedure and Troubleshooting.doc":[http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure\\_and\\_Troubleshooting.doc?1312282214](http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure_and_Troubleshooting.doc?1312282214) **C. Third option:** Seamless cloning using restriction free methods can be used to insert DNA sequences at any place of MultiLabel vectors. Add a 15–20 bases 5'-tail to the primers containing DNA sequences that are homologous to the sequences flanking the site of insertion into the MultiLabel vector. The described primers bind to the 3' end of the CMV promoter (RF-CMV-for) and to the 5' end of the poly A signal (RF-PolyA-back, Fig 2C). This method has two PCR steps. In the first PCR the gene of interest is amplified and this PCR fragment replaces in the second step the linker of the MultiLabel vector. See the attached document for details on how to do this. "Procedure and Troubleshooting.doc":[http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure\\_and\\_Troubleshooting.doc?1312282214](http://www.nature.com/protocolexchange/system/uploads/1864/original/Procedure_and_Troubleshooting.doc?1312282214) **CRITICAL STEPS** Steps 1Ai, 1Bi, 1Ci, and 1Civ It is critical to use a high fidelity proof-reading polymerase with a low error rate. We use Phusion high-fidelity DNA polymerase in 2× HF buffer, but the use of other polymerases is possible. The use of the wax beads reduces non-specific amplification during the PCR. Other hot start methods than wax beads are applicable but more

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PCR synthesized strand. Otherwise methylated parental DNA and hemimethylated hybrids would give rise to high background after transformation. **\*\*2|\*\*** Use 0.5 µl of ligation or restriction free reaction to transform competent E. coli cells (TOP10 or DH10β for acceptor plasmids or BW23474 for donor plasmids) and select on LB agar plates containing the appropriate antibiotic for selection (depending on the vector of choice; see Table 1). Incubate overnight at 37°C. **\*\*▲ CRITICAL STEP\*\*** The correct bacterial strain has to be chosen for transforming cloning products. Donor plasmids and derivatives contain a conditional origin of replication derived from R6Kγ and have to be propagated in cell strains expressing the pir gene such as BW23474. Acceptor plasmids and derivatives can be propagated in common laboratory bacterial strains (for example, TOP10 or DH10β). **TROUBLESHOOTING \*\*3|\*\*** Pick 2-10 white colonies (see Supplementary Fig. 2A) and inoculate small scale cultures in LB media containing the appropriate antibiotic. The isolated plasmids should then be analyzed by restriction mapping using restriction enzymes. **\*\*▲ CRITICAL STEP\*\*** Phusion polymerase has a low error rate. Nonetheless, it is advisable to verify the inserted gene by sequencing. **\*\*Creating multigene expression plasmids\*\* \*\*4|\*\*** Set up plasmid assembly reactions for 3 MultiLabel vectors in a 10 µl volume. ddH<sub>2</sub>O: – 2 µl 10× Cre reaction buffer: – 1 µl Acceptor plasmid (1 µg): – 2 µl Donor plasmid 1 (1 µg): – 2 µl Donor plasmid 2 (1 µg): – 2 µl Cre recombinase: – 1 µl Incubate for 1 hour at 37°C. **\*\*▲ CRITICAL STEP\*\*** Cre recombinase is a very sensitive enzyme; therefore DNA should be purified with an anion exchange kit. One or two donor plasmids can be routinely assembled with one acceptor plasmid. The third and fourth donor plasmids should be inserted in a sequential manner with triple-assembled and four-assembled multigene plasmid, respectively. **\*\*5|\*\*** Use 0.5 µl of the Cre recombinase reaction to transform competent E. coli cells (for example, TOP10 or DH10β). Plate the cells on LB agar plates containing appropriate combination of antibiotics (Supplementary Fig. 2B, Table 1). Incubate overnight at 30°C. **\*\*6|\*\*** Pick 2-10 colonies to grow small scale cultures for plasmid isolation in LB media containing the corresponding antibiotics. The isolated plasmids should then be analyzed by restriction mapping using appropriate restriction enzymes. **▣ TROUBLESHOOTING ▣ PAUSE POINT** Plasmids can be stored at 4°C. **\*\*Generation of stable cell lines\*\* \*\*Linearization of the multigene plasmid\*\*** Several acceptor vectors contain a recognition site for the homing endonuclease I-SceI (Supplementary Figure 1). If one of your genes of interest contains a I-SceI restriction site, the BstZ17I restriction enzyme can be used to linearize the multigene vector. The BstZ17I restriction site is localized on the border between Kanamycin and FRT regions and will not interfere with mammalian expression. **\*\*7|\*\*** Set up preparative restriction digestion reaction in 50 µl volume each: MultiLabel multigene vector (50 µg) – x µl 10× digestion buffer (Tango) – 5 µl I-SceI restriction enzyme – 1 µl ddH<sub>2</sub>O – till 50 µl Incubate for 16h at 37°C. **\*\*8|\*\*** Run an analytic agarose gel to check if the multigene vector is linearized completely. Purify the linearized multigene plasmid using the QIAprep PCR purification kit or phenol-chloroform extraction followed by ethanol precipitation. **\*\*▲ CRITICAL STEP\*\*** It is very important that the multigene plasmid is completely linearized prior transfection into the mammalian cells. **\*\*9|\*\*** Measure the concentration of the linearized multigene plasmid. **\*\*Preparation of mammalian cells for transfection in 10cm tissue culture plate\*\*** Note that this is our lab standard cell culture protocol for transfection of HEK293 and PAE cells. The protocol may need some adaptations or other cell types. **\*\*10|\*\*** Plate HEK293 or PAE cells 6-9 hours prior transfection in 10cm dishes. Cells should be 30-40% confluent prior to transfection. **\*\*▲ CRITICAL STEP\*\*** From now on, all steps should be performed in a sterile hood. **11|** Mix 5 µg linearized multigene plasmid with 7.5 µl FuGENE HD transfection reagent in 250 µl serum-free medium (Opti-MEM I). Incubate for 20 min at room temperature before adding to the plated cells. **\*\*▲ CRITICAL STEP\*\*** In addition to FuGENE HD, there are several other transfection reagents which can be used (for example, PEI or calcium phosphate). The choice depends on the used cell type. **\*\*Selection of stable transfectants\*\* \*\*12|\*\*** Cells are passed into 2-4 10 cm plates 40 h after transfection in different dilutions (1:2, 1:5, and 1:20). Add selection drug one day after replating (Table 1). Replace medium once or twice per week, always add drug. Colonies are visible within 14-21 days and should be picked (clone rings with grease for PAE cells or filter cloning discs (Sigma) for HEK293 cells). Transfer colonies to 1cm wells and grow in presence of selection drug. Pass into 3.5 or 6cm plates and freeze aliquots.

## Troubleshooting

**\*\*PROBLEM\*\* \*\*Steps 1Av, 1Bv, and 1Cv\*\*** There is no PCR product or multiple unspecific bands. **\*\*SOLUTION\*\* \*\*Steps 1Av, 1Bv, and 1Cv\*\*** Use the Phusion GC Buffer for those experiments where amplification with HF Buffer has failed. The GC Buffer can improve the performance of Phusion DNA Polymerase on some difficult or long templates, i.e. GC rich templates or those with complex secondary structures. Alternatively, several PCR reactions can be pooled to obtain more PCR product. If non-specific multiple bands are present, annealing temperature may have to be optimized or hot start didn't work. **\*\*PROBLEM\*\* \*\*Steps 2\*\*** a) There is high background of parental plasmids after transformation. b) No transformants were obtained. **\*\*SOLUTION\*\* \*\*Steps 2\*\*** a) Digest 10–12h or overnight with 20–40 units DpnI enzyme. b) More colonies can be obtained by using highly competent commercial cells. PCR fragments are sometimes difficult to cut and ligate. But they can be cloned easily into the pGEM-T vector (Promega) and purified from there as a fragment. Please note that Phusion Polymerase does not generate A overhangs. Therefore an additional incubation with Taq

polymerase prior to ligation is necessary. **PROBLEM** **Steps 6** a) No transformants were obtained. b) Plasmids can not be cut or are only partially cut by restriction enzymes. **SOLUTION** **Steps 6** a) The transformation efficiency of fusions can be increased by culturing the transformed cells overnight at 30°C in liquid culture containing appropriate antibiotics prior to plating and/ or the use of highly competent commercial cells. Antibiotic concentrations were reduced to 50% when 4 or 5 plasmids were assembled. b) Rearrangement or denaturation of plasmids during growth can be eliminated by culturing the bacteria in LB medium at 30°C rather than at 37°C.

## References

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## Figures



**Figure 1**

Workflow of the method.



**Figure 2**

Strategies to obtain expression vectors. (A) Existing expression cassettes of the pcDNA- (Invitrogen) or pEGFP-type (Clontech) can be amplified by PCR with general primers CMV-Ascl-for and PolyA-Pacl-back. The PCR fragment is then digested with Ascl and Pacl, and cloned in the desired backbone. Sequences of restriction sites are marked in bold. (B) All destination vectors contain two recognition sites for SapI. Note that they generate non-cohesive ends, therefore directed cloning is possible. Coding regions are amplified by PCR with primers SapI-for and SapI-back. The PCR fragment is digested with SapI and then ligated in a SapI digested MultiLabel vector. Sequence of restriction sites are marked in bold, start and stop codons are in red. (C) A gene of interest can be inserted into the MultiLabel vector by restriction enzyme free method. PCR primers RF-for and RF-back contain 18 bases homologous to the sequences of the MultiLabel insertion site and 15-20 bases complementary sequence to the 5' and 3' ends of the gene of interest. Sequences of homologous regions to the 3' end of the CMV promoter and the 5' end of the Poly A region are marked in green.

**Table 1 | Working concentration of antibiotics**

Bacterial selection	concentration	
Ampicillin	50 µg/ml	
Kanamycin	25 µg/ml	NOTE: Reduce antibiotics concentration to 50% when 4 or 5 plasmids are assembled.
Spectinomycin	50 µg/ml	
Gentamycin	4 µg/ml	
Chloramphenicol	4 µg/ml	

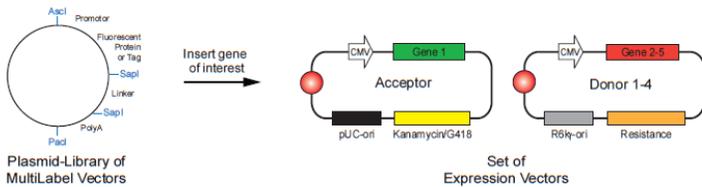
Mammalian cell selection	concentration
G418	400 µg/ml
Zeocin	500 µg/ml
Hygromycin	500 µg/ml

**Figure 3**

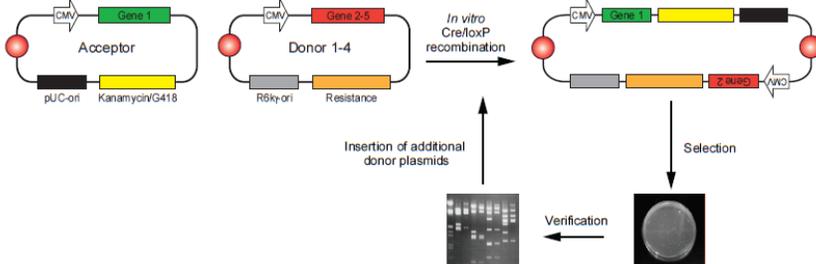
Table 1 Working concentration of antibiotics

Figure 1: Workflow of the method

**A) Construction of MultiLabel vectors with a single expression cassette (Steps 1–3)**



**B) Creating multigene expression plasmids (Steps 4–6)**



**C) Generation of stable cell lines (Steps 7–13)**

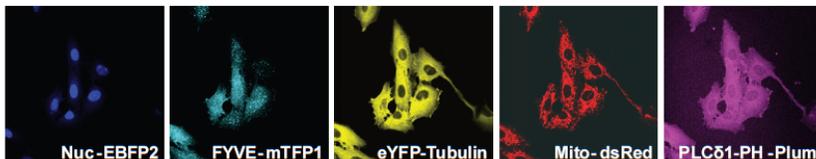
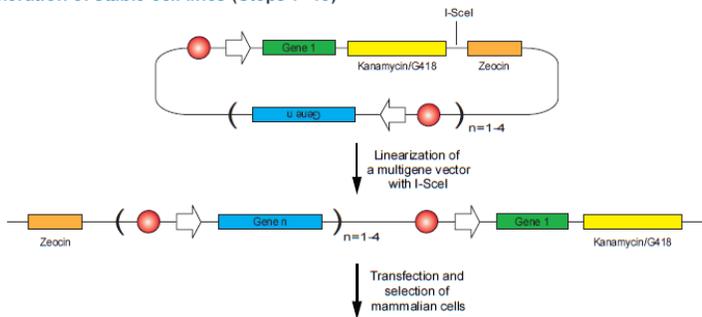
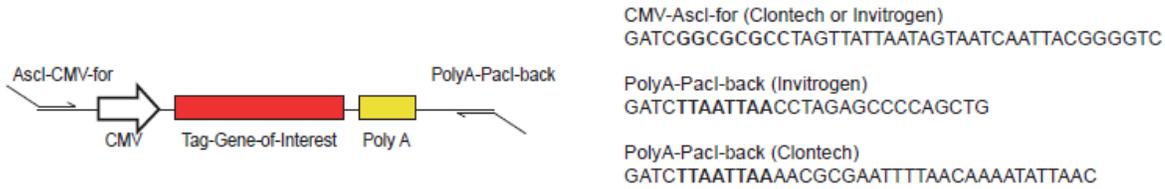


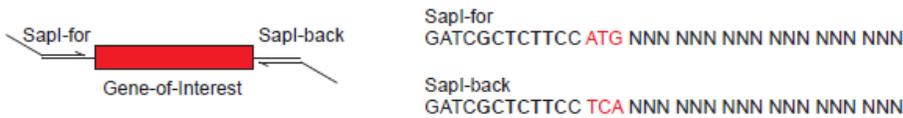
Figure 1 Workflow of the method

## Figure 2: Strategies to obtain expression vectors

### A) Usage of existing expression vectors



### B) Fusion proteins with an N-terminal fluorescent tag



### C) Restriction enzyme free insertion of an untagged gene of interest

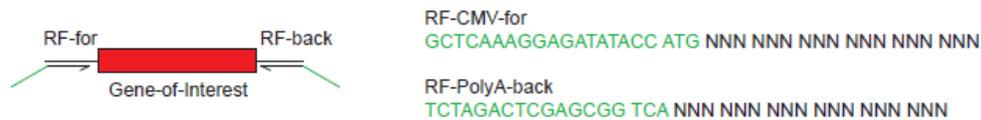


Figure 5

Figure 2 Strategies to obtain expression vectors. (A) Existing expression cassettes of the pcDNA- (Invitrogen) or pEGFP-type (Clontech) can be amplified by PCR with general primers CMV-Ascl-for and PolyA-Pacl-back. The PCR fragment is then digested with Ascl and Pacl, and cloned in the desired backbone. Sequences of restriction sites are marked in bold. (B) All destination vectors contain two recognition sites for SapI. Note that they generate non-cohesive ends, therefore directed cloning is possible. Coding regions are amplified by PCR with primers SapI-for and SapI-back. The PCR fragment is digested with SapI and then ligated in a SapI digested MultiLabel vector. Sequence of restriction sites are marked in bold, start and stop codons are in red. (C) A gene of interest can be inserted into the MultiLabel vector by restriction enzyme free method. PCR primers RF-for and RF-back contain 18 bases homologous to the sequences of the MultiLabel insertion site and 15-20 bases complementary sequence to the 5' and 3' ends of the gene of interest. Sequences of homologous regions to the 3' end of the CMV promoter and the 5' end of the Poly A region are marked in green.

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