# MultiMam ${ }^{\text {TM }}$ Transient 

## Transient Multiprotein Expression in Mammalian Cells



# GENEVA BIOTECH 

User Manual

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## MultiMam ${ }^{\text {TM }}$ Transient Kit Contents

- Plasmid acceptor vectors
pACEMam1, pACEMam2; approx. $5 \mu \mathrm{~g}$ DNA per vial (in buffer solution)
keep at $4^{\circ} \mathrm{C}$ for short-term storage and in a freezer at $-20^{\circ} \mathrm{C}$ or lower for medium- and long-term storage (take care to avoid repeated freeze-thaw cycles, e.g. by aliquotting DNA prior to freezing)
- Plasmid donor vectors
pMDC, pMDK, pMDS; approx. $5 \mu \mathrm{~g}$ DNA per vial (in buffer solution)
keep at $4^{\circ} \mathrm{C}$ for short-term storage and in a freezer at $-20^{\circ} \mathrm{C}$ or lower for medium- and long-term storage (take care to avoid repeated freeze-thaw cycles, e.g. by aliquotting DNA prior to freezing)
- E. coli strains as agar stabs
pirHC, pirLC cells ${ }^{+}$
For propagation and amplification of donor multigene expression constructs or donor-donor fusions

Keep agar stabs at $4^{\circ} \mathrm{C}$ or at RT; do not freeze! We recommend to immediately prepare stocks from streaked bacterial colonies (see p. 26).
${ }^{\dagger}$ E. coli strains expressing the pir gene for propagation of donor vectors (any other strain with pir background can be used as well). LC: low copy number propagation, HC: high copy number propagation of plasmids with $\mathrm{R} 6 \mathrm{~K} \gamma$ origin.

## Reagents to be supplied by the user (see also Section D. Protocols)

- Restriction enzymes and Homing endonucleases PI-Scel and I-Ceul
- Mammalian cells, e.g. HEK293, CHO, etc.
- T4 DNA ligase
- Cre recombinase
- Standard E. coli strains for cloning (such as TOP10, DH5 $\alpha$, HB101 etc.)
- Standard laboratory buffers, solutions, media and equipment for bacterial and mammalian cell culture, transformation etc.
- Commercially available transfection reagents, e.g. FuGENE ${ }^{\circledR}$ (Roche), jetPEI ${ }^{\text {TM }}$ (Polyplus transfection), etc. or an apparatus for electroporation
- Antibiotics


## B. Introduction

## Cellular interaction networks and protein complexes

In 1998, Bruce Alberts confronted conventional thinking that predicated on the action of individual proteins on Beadle and Tatum's one-gene/one-enzyme hypothesis (published in 1941) which for decades had shaped much of biological research. Instead, Alberts asked us to direct our focus to a modular cellular machinery composed of protein complexes (Alberts, 1998).

Proteins are the physical representatives of the information encoded by their corresponding genes and mRNAs. They are themselves embedded into a tightly and intricately regulated DNA-RNA-network (Vidal et al., 2011). These proteins determine many structural and physiological properties of cells but rarely act in isolation to mediate their effects. More often than not they will have multiple partners - not only proteins but also nucleic acids and small molecules - which they bind or bind to or associate with in larger complexes. Whether you look at replication, transcription, translation, transport processes across internal and external membranes, signaling events, etc. - protein complexes come into play in all of these processes. More importantly, such complexes - if disrupted by mutations or the like - also engender often severe physiological deficits (Ehmsen et al., 2002; Vidal et al., 2011). Some of these complexes will, by their functional nature, either be long-lived ("stable") or transitory. Fleeting interaction of proteins, e.g. in cell signaling, will result in only minute amounts of a protein complex that usually also exists for only a limited period of time.

Deconvoluting this social life of the cell (Robinson et al., 2007) is a daunting task but has been tackled with high resolution imaging and analysis techniques (cryoEM, X-ray crystallography, NMR, mass spectroscopy, etc.). Extensive bioinformatics work-up and computer modeling support the experimental structural biology work and contribute to solving complex multi-subunit assemblies down to the atomic level (e.g. Imasaki et al., 2011). All these results enter into a better understanding of molecular interactions between proteins and other macromolecules, now known as the interactome (Figeys, 2008; Charbonnier et al., 2008) and their effects on the biological system of the cell.

## Multiprotein expression tools

Various heterologous systems have been developed for the major production/host organisms E.coli, yeast, insect and mammalian cells. While sophisticated system for expressing individual proteins exist, the repertoire of tools for multiprotein expression to date is rather limited (e.g, Bieniossek et al. 2009; Trowitzsch et al., 2010), especially for mammalian cells.

This cell culture of transgene-expressing cells has become one of the mainstays of functional investigations in cellular physiology and biochemistry. Co-transfection, whether by biochemical or physical means or through viruses, still is the method of choice when it comes to delivering genes of interest into mammalian cells. Co-transfection often fails to warrant uniform and constant expression of all vectors in one transfection experiment. Stable transfection remedies this problem to a certain degree but is cumbersome and requires multiple rounds of selection and re-culturing to yield uniform and stable clones.

Vector systems that enable uniform transient and, also stable transfection of multiple genes are in demand for mammalian cells. This manual introduces a set of novel mammalian transfer vectors that specifically enables transient multiprotein expression. Our sister technology MultiMam ${ }^{\text {rM }}$ Stable enables generation of stable multiprotein-expressing cell lines.

The role of protein interaction networks (the so-called interactome) has become an intense focus of biological research efforts in the post-genomic era. Many of the identified multiprotein complexes are expressed at only low abundance in their native cells. This makes analysis of their structure difficult, but this can be remedied by using recombinant technologies to facilitate large-scale heterologous protein production. Currently, recombinant expression methods require a disproportionate investment in both labor and materials prior to multiprotein expression, and, once expression has been established, provide little or no flexibility for rapidly altering the multiprotein components, which is a prerequisite for revising expression studies. The mammalian expression system introduced here (see also Vijayachandrain et al., 2011) boasts two major advances that are instrumental in fully exploiting the potential of this heterologous protein production system:

1) New transfer vectors (pACEMam1, pACEMam2, pMDC, pMDK, pMDS; see Figure 1) that contain a homing endonuclease-based multiplication module. These vectors greatly facilitate modular combination of heterologous genes (in their respective gene expression cassettes) with a minimum requirement for unique restriction sites (BstXI). Strong viral/mammalian promoters (currently CMV and the hybrid CAG promoters) can be exchanged in our vectors for other promoter sequences if desired. Likewise, terminator sequences (currently SV40, rabbit $\beta$-actin) can be substituted as required.


Figure 1: Schematic representation of the MultiMam ${ }^{\text {TM }}$ Transient acceptor and donor vectors. More detailed vectors maps and sequence information can be found in Chapter $C$ and in the Appendix.
2) New protocol for rapid generation of multigene expression constructs via Cre-LoxP recombineering. The resulting multigene fusion can then be transfected directly into mammalian cells for transient expression. This protocol can be used to integrate multigene
cassettes with coding sequences for multiprotein complex subunits but also to integrate specific enzymes (kinases, acetylases etc.) for modifying the proteins under investigation.


Figure 2a: Schematic overview of the MultiMam ${ }^{\text {™ }}$ Transient system and its application.
Genes of interest are assembled into multigene expression cassettes using the multiplication module present on the donor ( $\mathrm{pMDC}, \mathrm{pMDK}, \mathrm{pMDS}$ ) and acceptor vectors ( $\mathrm{pACEMam1}$, pACEMam2). Acceptor-donor fusions can then be generated by Cre-LoxP recombination. These multigene fusions contain one Acceptor and one to several Donor vectors, each with one or
several genes of interest (here A-H). Desired Acceptor-Donor combinations are identified by transformation into E.coli and subsequent antibiotic selection. LoxP sites in the acceptor-donor fusions have been omitted for reasons of clarity.


Figure 2B: Generation of multigene donor constructs through Cre-Lox fusion.
As indicated in Figure 2A, donor multigene expression cassette constructs can also be generated by Cre-Lox recombination. Individual or multiple gene cassettes are cloned into the multiple cloning site via standard restriction-ligation cloning or, when introducing multiple gene cassettes, homing endonuclease /BstXI cloning. The gene cassettes harbored on different donor vectors are then merged into a single vector construct via Cre-Lox recombination. This construct will differ from the multigene constructs in Figure 2a with respect to selective markers. While the multigene construct in fig. 2A carries only one antibiotic resistance marker, the construct in fig. 2B will carry three, one from each donor vector. This will allow selection of multigene constructs with higher stringency by subjecting the constructs to a multi-antibiotic selection regimen (refer to protocol 2). LoxP sites in the donor fusion have been omitted for reasons of clarity.

## C. New Tools for Multigene Applications in Mammalian Cells

## C.1. Transfer vectors: the Acceptor-Donor recombineering system.

The Acceptor vectors pACEMam1 and pACEMam2 contain multiple cloning sites (MCS; see appendix) flanked by either a CMV or CAG promoter to drive high-level expression in mammalian cells Wherever necessary, appropriate polyA signal sequences are available (SV40 late for pACEMam1).

A multiplication module $M$ - defined by the homing endonuclease site I-Ceul and a corresponding BsXI site (see Figure 3) - allows integration of multiple gene cassettes (ORFs and associated regulatory regions).
Figure 3: Circle map representation of Acceptor
vectors
(a) pACEMam1 (3443 bp),

The Donor vectors pMDC, pMDS, pMDK are similar to the acceptor vectors with respect to their over-all design. The multiple cloning site is bracketed by a multiplication element (in this case, PI Scel / BstXI) to enable concatenation of inserts between the different donor vectors. Vectors also contain a LoxP incomplete inverted repeat to create acceptor-donor or donor-donor fusions. The vectors contain "tell-tale" resistance markers (pMDC: chloramphenicol, pMDK: kanamycin, pMDS: spectinomycin) and, importantly, a conditional $\mathrm{R} 6 \mathrm{~K} \gamma$ origin of replication which makes propagation of the donor vectors dependent on the expression of the pir gene in the prokaryotic host (such as the pirLC and pirHC cells contained in the kit).

Figure 4: Circle map representation of Donor vectors a) a) pMDC, b) pMDK, c) pMDS.

Circle maps show promoters (CMV, CAG), terminators (SV40, rabbit $\beta$-globin), multiple cloning sites (MCS), the incomplete inverted repeat for cre-lox site-specific recombination (LoxP) and resistance markers (chloramphenicol, kanamycin, and spectinomycin, respectively). Genes of interest are cloned into the MCS using unique restriction sites. The multiplication module consists of the homing endonuclease site PIScel and the restriction endonuclease site BstXI. All donor vectors host a conditional $\mathrm{R} 6 \mathrm{~K} \gamma$ origin of replication.
b)


c)


The MultiMam ${ }^{\text {TM }}$ Transient system vectors in their current form do not contain DNA sequences that code for affinity tags (that will facilitate purification or solubilization of the protein(s) of interest). Tags that are typically used are C- or N-terminal oligohistidine tags, with or without protease cleavage sites for tag removal. They can be introduced by designing the respective PCR primers used for amplification of the genes of interest. We recommend outfitting Donors or Acceptors of choice with any custom tag that is favored in individual user laboratories prior to inserting recombinant genes of interest. This is best done by using a design that will, after tag insertion, still be compatible with the recombination-based principles of MultiMam ${ }^{\text {M }}$ Transient system usage.

The same holds true for reporter genes, most notably fluorescent proteins that are commonly used in protein localization or protein interaction studies. These can also be fused to your protein under investigation using PCR techniques.

## C.2. Generating multigene expression cassettes

## C.2.1. Using the homing endonuclease/BstXI multiplication module

The acceptor and donor vectors are suited for generating multigene expression cassettes from individual gene expression cassettes (complete with regulatory regions such as promoter and terminator) via a multiplication module bracketing the multiple cloning site (MCS). All MultiMam ${ }^{\text {M }}$ Transient vectors contain a homing endonuclease (HE) site and a correspondingly designed BstXI site that together bracket the MCS. Homing endonucleases have long recognition sites (20-30 base pairs or more). Although not all equally stringent, homing endonuclease sites are very likely unique in the context of even large plasmids, or, in fact, entire genomes.

The logic of multiplication is illustrated below. The homing endonuclease site can be used to insert entire expression cassettes into a vector that already contains one gene or several genes of interest as separate expression cassettes. The only prerequisite for assembling multigene expression cassettes is that the homing endonucleases and restriction enzymes used for multiplication (I-Ceul/PIScel and BstXI) are unique, which can be easily accomplished, for instance by site-directed mutagenesis prior to multigene cassette assembly. First, individual genes are cloned into the multiple cloning sites of the acceptor and donor vectors. The entire expression cassette, including promoter and terminator, is then excised by I-Ceul / BstXI (acceptors) or PI-Scel / BstXI (donors) digestion. The resulting fragment is placed into the multiplication module of another acceptor or donor vector containing single or multiple gene cassettes. The restriction sites involved are eliminated in the process and multiplication can be repeated iteratively using the module present in the inserted cassette. Moreover, promoter and terminator sequences can be easily modified if desired using appropriate restriction sites in our vectors.

Please note that multiplication cannot be accomplished from donors to acceptors and vice versa since the overhangs generated by endonuclease digestion are incompatible.


Figure 5: Assembling individual gene cassettes into multigene expression cassettes. The logic of multiplication is shown schematically. The expression cassette containing the gene of choice (denoted as GOI2 in this case) is excised by digestion with the homing endonuclease (red box) and BstXI (green box). For acceptors vectors, I-Ceul is the homing endonuclease of choice, and for donor vectors PI-Scel. The plasmid vector harboring the GOI1-cassette only needs to be linearized with BstXI. The homing endonucleases produce cohesive ends that are compatible with the ends generated by the BstXI digest. Upon insertion of GOI2 into the target vector, a homing endonuclease/BstXI hybrid restriction site is created that can then cannot be cut by either enzyme (crossed-out red/green box) while the 3'-BstXI site is regenerated. The same procedure can be repeated over and over as exemplified by the integration of GOI3. This cycling logic can be used to generate multigene assemblies. Note that the promoters and terminators are not explicitly shown for reasons of clarity.


Figure 6: Combining multigene expression cassettes. Different multigene expression cassettes can be combined into one expression construct following the same logic that applies to the generation of multigene expression cassettes from individual gene cassettes (Figure 4). The 5' homing endonuclease recognition site (filled red box) will be preserved if GOI1 has been introduced by conventional restriction cloning into the MCS. Promoters and terminators are not explicitly shown for reasons of clarity but flank the GOIs in every individual gene expression cassette.

## C.2.2. Multigene construction using Cre-Lox recombination

Cre recombinase is a member of the integrase family (Type I topoisomerase from bacteriophage P1). It recombines a 34 bp loxP site in the absence of accessory protein or any auxiliary DNA sequence. The loxP site is comprised of two 13 bp recombinase-binding elements arranged as inverted repeats which flank an 8 bp central region where cleavage and the ligation reaction occur.

The site-specific recombination mediated by Cre recombinase involves the formation of a Holliday junction (HJ). The recombination events catalyzed by Cre recombinase depend on the location and relative orientation of the loxP sites. Two DNA molecules, for example an acceptor and a donor plasmid, containing single loxP sites will be fused. Furthermore, the Cre recombination is an equilibrium reaction with $20-30 \%$ efficiency in recombination. This provides useful options for multigene combinations for multiprotein complex expression.

| 13bp | 8bp | 13 bp |
| :---: | :---: | :---: |
| 5'...ATAACTTCGTATA | GCATACAT | TATACGAAGTTAT...3' |
| 3'...TATTGAAGCATAT $^{\prime}$ | CGTATGTA | ATATGCTTCAATA...5' |
| inverted repeat | Spacer | inverted repeat |

Figure 7: LoxP imperfect inverted repeat

In a reaction where several DNA molecules such as donors and acceptors are incubated with Cre recombinase, the fusion/excision activity of the enzyme will result in an equilibrium state where single vectors (educt vectors) and all possible fusions coexist. Donor vectors can be used with acceptors and/or donors, and vice versa. Higher order fusions are also generated where more than two vectors are fused. This is shown schematically in Figure 8.

The fact that Donors contain a conditional origin of replication that depends on a pir (pir positive) background now allows for selecting out from this reaction mix all desired Acceptor-Donor(s) combinations. For this, the reaction mix is used to transform pir negative strains (TOP10, DH5 $\alpha$, HB101 or other common laboratory cloning strains). Then, Donor vectors will act as suicide vectors when plated out on agar containing the antibiotic corresponding to the Donor encoded resistance marker, unless fused with an Acceptor. By using agar with the appropriate combinations of antibiotics, all desired Acceptor-Donor fusions can be selected for.


Figure 8: Cre and De-Cre reaction pyramid
Cre-mediated assembly and disassembly of pACEMam1, pMDK, and pMDS vectors are shown in a schematic representation (left). LoxP sites are shown as red circles, resistance markers and origins are labeled. White arrows stand for the entire expression cassette (including promoter, terminator and gene integration/multiplication elements) in the MultiMam ${ }^{\text {rM }}$ Transient vectors. Not all possible fusion products are shown for reasons of clarity. Levels of multi-resistance are indicated (right column).

## C.2.3. Combining HE/BstXI cycling and Cre-Lox recombination

Of course, both methods can also be combined to generate multiple gene-expression cassette constructs. To this end, you can introduce multiple gene cassettes with the homing endonuclease/BstXI protocol into different Acceptor/Donor vectors and then fuse these using the CreLox modules (illustrated in Figure 2a).

## D. Protocols

## D. 0 Introductory remarks

Please note that the bacteria in the agar stabs have not been made competent for transformation. If you wish to use them to transform your constructs, you will have to prepare competent cells. This applies specifically to the pirHC and pirLC strains used to maintain donor constructs. You may follow your preferred protocol for preparing chemically or electrocompetent cells, e.g. Inoue et al. (1990) or variations of this protocol, or standard protocols as described in Current Protocols in Molecular Biology or Sambrook and Russell: Molecular Cloning (3 ${ }^{\text {rd }}$ edition, 2001, or older versions).

## D. 1 Cloning into pACEMam or pMDx transfer vectors

Reagents:<br>Restriction endonucleases<br>DNA ligase<br>E. coli competent cells<br>Antibiotics: Chloramphenicol, Gentamycin, Kanamycin, Spectinomycin

The genes of choice are cloned using standard cloning procedures into the multiple cloning sites MCS (see Supplementary Information) of pACEMam1/2 and pMDC, pMDK, pMDS. Ligation reactions for pACEMam derivatives are transformed into standard E. coli cells for cloning (such as TOP10, DH5 $\alpha$, HB101) and plated on agar containing gentamycin ( $7 \mu \mathrm{~g} / \mathrm{ml}$ ). Ligation reactions for pIDx derivatives are transformed into E. coli cells expressing the pir gene (pirHC and pirLC from this kit - in this case you will need to make the cells electro- or chemically competent first; other strains, e.g. BW23473, BW23474) and plated on agar containing chloramphenicol (pMDC; $25 \mu \mathrm{~g} / \mathrm{ml}$ ), kanamycin (pMDK; 50 $\mu \mathrm{g} / \mathrm{ml}$ ) or spectinomycin (pMDS; $50 \mu \mathrm{~g} / \mathrm{ml}$ ). Correct clones are selected based on specific restriction digestion patterns and DNA sequencing of the inserts.

## D. 2 Multiplication by using the HE and BstXI sites

MultiMam ${ }^{\text {TM }}$ Transient donor vectors contain a recognition site for the homing endonuclease PI-Scel (fig. 3). Upon cleavage, this HE site yields a 3' overhang with the sequence -GTGC. Acceptor vectors contain the homing endonuclease site l-Ceul (see fig. 2), which upon cleavage will result in a 3'
overhang of -CTAA. On acceptors and donors, the respective HE site precedes the MCS (see Figure 2). The 3' end of the MIE contains a specifically designed BstXI site, which upon cleavage will generate a matching overhang. The basis of this is the specificity of cleavage by BstXI. The recognition sequence of BstXI is defined as CCANNNNN'NTGG (the apostrophe marks the position of the phosphodiester link cleavage). The residues denoted as N can be chosen freely. Donor vectors thus contain a BstXI recognition site with the sequence CCATGTGC'CTGG, and Acceptor vectors contain CCATCTAA'TTGG. The overhangs generated by BstXI cleavage in each case will match the overhangs generated by HE cleavage. Note that Acceptors and Donors have different HE sites.

The recognition sites are not symmetric. Therefore, ligation of a $\mathrm{HE} / \mathrm{BstXI}$ digested fragment into a HE site of an MultiMam ${ }^{\text {TM }}$ Transient vector will be (1) directional and (2) result in a hybrid DNA sequence where a HE halfsite is combined with a BstXI half site (see Figure 5). This site will be cut by neither the HE nor BstXI. Therefore, in a construct that has been digested with a HE, insertion by ligation of $\mathrm{HE} / \mathrm{BstXI}$ digested DNA fragment containing an expression cassette with one or several genes will result in a construct which contains all heterologous genes of interest, enveloped by an intact HE site in front, and a BstXI site at the end. Therefore, the process of integrating entire expression cassettes by means of $\mathrm{HE} / \mathrm{BstXI}$ digestion and ligation into a HE site can be repeated iteratively.

## D. 2 Protocol 1. Multiplication by using homing endonuclease/BstXI.

Reagents required:<br>Homing endonucleases PI-Scel, I-Ceul<br>10x Buffers for homing endonucleases<br>Restriction enzyme BstXI (and 10x Buffer)<br>T4 DNA ligase (and 10x Buffer)<br>E. coli competent cells<br>Antibiotics

Step 1: Insert preparation
Restriction reactions are carried out in $40 \mu \mathrm{I}$ reaction volumes, using homing endonucleases PIScel (Donors) or I-Ceul (Acceptors) as recommended by the supplier.

Acceptor or donor plasmid ( $\geq 0.5 \mu \mathrm{~g}$ ) in $\mathrm{ddH}_{2} \mathrm{O} \quad 32 \mu \mathrm{l}$
10x restriction enzyme buffer
10 mM BSA
PI-Scel (Donors) or l-Ceul (acceptors)
Reactions are then purified using a PCR extraction kit or by acidic ethanol precipitation, and
subsequently digested with BstXI according to the supplier's recommendations.

HE digested DNA in ddH 2 O
10x restriction enzyme buffer
10 mM BSA
BstXI
Gel extraction of insert(s):
Processed insert is then purified by agarose gel extraction using commercial kits (Qiagen,
Macherey Nagel etc). Elution of the extracted DNA in the minimal volume defined by the
manufacturer is recommended.

## Step 2: Vector preparation

Restriction reactions are carried out in $40 \mu \mathrm{l}$ reaction volumes, using homing endonucleases PI Scel (Donors) or I-Ceul (Acceptors) as recommended by the supplier.
Acceptor or donor plasmid ( $\geq 0.5 \mu \mathrm{~g}$ ) in $\mathrm{ddH}_{2} \mathrm{O}$
$33 \mu \mathrm{l}$
10x Restriction enzyme buffer $4 \mu \mathrm{l}$
10 mM BSA $2 \mu \mathrm{l}$
BstXI $2 \mu \mathrm{l}$

Reactions are then purified by PCR extraction kit or acidic ethanol precipitation, and next treated with intestinal alkaline phosphatase according to the supplier's recommendations. Dephosphorylation is performed to minimize vector re-annealing and to increase integration of the insert.

HE digested DNA in ddH $_{2} \mathrm{O} \quad 17 \mu \mathrm{l}$
10x Alkaline phosphatase buffer $2 \mu \mathrm{l}$
Alkaline phosphatase $\quad 1 \mu$ l

Gel extraction of vector:
Processed vector is then purified by agarose gel extraction using commercial kits (Qiagen, MachereyNagel etc). Elution of the extracted DNA in the minimal volume defined by the manufacturer is recommended.

Step 3: Ligation
Ligation reactions are carried out in $20 \mu$ reaction volumes:
$\mathrm{HE} /$ Phosphatase treated vector (gel extracted) $4 \mu \mathrm{l}$
$\mathrm{HE} / \mathrm{BstXI}$ treated insert (gel extracted) $14 \mu \mathrm{l}$
10x T4 DNA Ligase buffer $\quad 2 \mu \mathrm{l}$

T4 DNA Ligase $\quad 0.5 \mu \mathrm{l}$
Ligation reactions are performed at $25^{\circ} \mathrm{C}$ for 1 h or at $16^{\circ} \mathrm{C}$ overnight.

## Step 4: Transformation

Mixtures are next transformed into competent cells following standard transformation procedures.
Ligation reactions for pACEMam1 and pACEMam2 derivatives are transformed into standard $E$. coli cells for cloning (such as TOP10, DH5 $\alpha, \mathrm{HB} 101$ ) and, after recovery, are plated on agar containing gentamycin ( $7 \mu \mathrm{~g} / \mathrm{ml}$ ).
Reactions for Donor derivatives are transformed into $E$. coli cells expressing the pir gene (such as BW23473, BW23474, or PIR1 and PIR2 from Invitrogen and, of course, pirLC and pirHC in this kit) and plated on agar containing chloramphenicol ( $25 \mu \mathrm{~g} / \mathrm{ml}, \mathrm{pMDC}$ ), kanamycin ( $50 \mu \mathrm{~g} / \mathrm{ml}, \mathrm{pMDK}$ ), or spectinomycin ( $50 \mu \mathrm{~g} / \mathrm{ml}$, pMDS).

Step 5: Plasmid analysis
Plasmids are cultured and correct clones selected based on specific restriction digestion and DNA sequencing of the inserts.

## D. 3 Cre-LoxP reaction of Acceptors and Donors

## D 3.1. Protocol 2: Cre-LoxP fusion of Acceptors and Donors

This protocol is designed for generating multigene fusions from Donors and Acceptors by Cre-LoxP reaction.

## Reagents:

Cre recombinase (from NEB or self-made)
Standard E. coli competent cells (pir strain)
Antibiotics
96-well microtiter plates
12 well tissue-culture plates (or Petri dishes) w. agar/antibiotics
LB medium

1. For a $20 \mu$ l Cre reaction, mix 1-2 $\mu \mathrm{g}$ of each educt in approximately equal amounts. Add $\mathrm{ddH}_{2} \mathrm{O}$ to adjust the total volume to $16-17 \mu \mathrm{l}$, then add $2 \mu \mathrm{l} 10 \mathrm{x}$ Cre buffer and 1-2 $\mu$ l Cre recombinase (1-2 U).
2. Incubate Cre reaction at $37^{\circ} \mathrm{C}$ (or $30^{\circ} \mathrm{C}$ ) for 1 hour.
3. Optional: load 2-5 $\mu$ l of Cre reaction on an analytical agarose gel for examination. Heat inactivation at $70^{\circ} \mathrm{C}$ for 10 minutes before gel loading is strongly recommended.
4. For chemical transformation, mix 10-15 $\mu$ l Cre reaction with $200 \mu$ l chemically competent cells. Incubate the mixture on ice for $15-30$ minutes. Then perform heat shock at $42^{\circ} \mathrm{C}$ for $45-$ 60 s.

Up to $20 \mu$ l Cre reaction ( 0.1 volumes of the chemically competent cell suspension) can be directly transformed into $200 \mu$ l chemical competent cells.

For electrotransformation, up to $2 \mu \mathrm{l}$ Cre reaction can be directly mixed with $100 \mu \mathrm{l}$ electrocompetent cells, and transformed by using an electroporator (e.g. BIORAD E. coli Pulser) at 1.8-2.0 kV.

Larger volumes of Cre reaction must be desalted by ethanol precipitation or via PCR purification columns before electrotransformation. The desalted Cre reaction mix should not exceed 0.1 volumes of the electrocompetent cell suspension.

The cell/DNA mixture can be immediately used for electrotransformation without prolonged incubation on ice.
5. Add up to $400 \mu \mathrm{l}$ of LB (or SOC) medium per $100 \mu \mathrm{l}$ of cell/DNA suspension immediately after the transformation (heat shock or electroporation).
6. Incubate the suspension in a $37^{\circ} \mathrm{C}$ shaking incubator overnight or for at least 4 hours (recovery period).

To recover multifusion plasmid containing more than 2 resistance markers, it is strongly recommended to incubate the suspension at $37^{\circ} \mathrm{C}$ overnight.
7. Plate out the recovered cell suspension on agar containing the desired combination of antibiotics. Incubate at $37^{\circ} \mathrm{C}$ overnight.
8. Clones from colonies present after overnight incubation can be verified by restriction digestion at this stage (refer to steps 12-16).

This quality control step should be carried out especially in the case that only one specific multifusion plasmid is desired.

For further selection by single antibiotic challenges on a 96 well microtiter plate, continue to step 9.

Several to many different multifusion plasmid combinations can be processed and selected in parallel on one 96 well microtiter plate.
9. For 96 well antibiotic tests, inoculate four colonies from each agar plate with different antibiotic combinations into approx. $500 \mu \mathrm{LB}$ medium without antibiotics. Incubate the cell cultures in a $37^{\circ} \mathrm{C}$ shaking incubator for 1-2 hours.
10. While incubating the colonies, fill a 96-well microtiter plate with $150 \mu \mathrm{l}$ antibiotic-containing LB medium (following Illustration 7). It is recommended to add coloured dye (positional marker) in the wells indicated.

A typical arrangement of the solutions, which is used for parallel selections of multifusion plasmids, is shown in Figure 9. The concept behind the 96 well plate experiment is that every cell suspension from single colonies needs to be challenged by all four single antibiotics for unambiguous interpretation.


Figure 9: 96 well analysis of Cre assembly
11. Add $1 \mu \mathrm{l}$ aliquots of pre-incubated cell culture (Step 9) to the corresponding wells. Then incubate the inoculated 96 well microtiter plate in a $37^{\circ} \mathrm{C}$ shaking incubator overnight at 180200 rpm .

Recommended: use parafilm or any other adhesive seal to wrap the plate to avoid drying out.
The remainder of the pre-incubated cell cultures can be kept at $4^{\circ} \mathrm{C}$ for further inoculations if necessary.
12. Select transformants containing desired multifusion plasmids based on antibiotic resistance, according to the combination of dense (positive) and clear (no growth) cell microcultures from each colony. Inoculate $10-20 \mu \mathrm{l}$ cell culture into 10 ml LB media with corresponding antibiotics. Incubate in a $37^{\circ} \mathrm{C}$ shaking incubator overnight.
13. Centrifuge the overnight cell cultures at 4000 g for $5-10$ minutes. Purify plasmid from the resulting cell pellets with common plasmid miniprep kits, according to manufacturer's recommendation.
14. Determine the concentrations of purified plasmid solutions by using UV absorption spectrophotometry (e.g. by using a NanoDrop ${ }^{\mathrm{TM}} 1000$ machine).
15. Digest $0.5-1 \mu \mathrm{~g}$ of the purified plasmid solution in a $20 \mu \mathrm{l}$ restriction digestion with appropriate endonuclease(s). Incubate under recommended reaction condition for approx. 2 hours.
16. Use 5-10 $\mu \mathrm{l}$ of the digestion for analytical agarose (0.8-1.2\%) gel electrophoresis. Verify plasmid integrity by comparing the experimental restriction pattern to a restriction pattern predicted in silico (e.g. by using program VectorNTI from Invitrogen or similar programs).

## D 3.2. Protocol 3. Deconstruction of fusion vectors by Cre

The following protocol is suitable for releasing any single educt from multifusion constructs (deconstruction). This is achieved by Cre-LoxP reaction, transformation and plating on agar with appropriately reduced antibiotic resistance level (cf. Figure 10). In the liberated educt entity, encoding genes can be modified and diversified. Then, the diversified construct is resupplied by Cre-LoxP reaction (C3.1).


Figure 10: 96 well analysis of Cre de-assembly

## Reagents:

Cre recombinase (and 10x Buffer)
E. coli competent cells
(pir ${ }^{+}$strains, pir strains can be used only when partially deconstructed Acceptor-Donor fusions are desired).

Antibiotics

1. Incubate approx. $1 \mu$ g multifusion plasmid with $2 \mu \mathrm{l} 10 \mathrm{x}$ Cre buffer, 1-2 $\mu \mathrm{l}$ Cre recombinase, add $\mathrm{ddH}_{2} \mathrm{O}$ to adjust the total reaction volume to $20 \mu \mathrm{l}$.
2. Incubate this Cre deconstruction reaction mixture at $30^{\circ} \mathrm{C}$ for 1 to 4 hour(s).
3. Optional: load 2-5 $\mu$ l of the reaction on an analytical agarose gel for examination.

Heat inactivation at $70^{\circ} \mathrm{C}$ for 10 minutes before gel loading is strongly recommended.
4. For chemical transformation, mix $10-15 \mu$ l De-Cre reaction with $200 \mu$ chemically competent cells. Incubate the mixture on ice for $15-30$ minutes. Then perform heat shock at $42^{\circ} \mathrm{C}$ for 45 60 s .

Up to $20 \mu \mathrm{I}$ De-Cre reaction ( 0.1 volumes of the chemical competent cell suspension) can be directly transformed into $200 \mu$ l chemically competent cells.
For electrotransformation, up to $2 \mu \mathrm{l}$ De-Cre reaction can be directly mixed with $100 \mu \mathrm{l}$ electrocompetent cells, and transformed by using an electroporator (e.g. BIORAD E. coli Pulser) at 1.8-2.0 kV.
Larger volume of De-Cre reaction must be desalted by ethanol precipitation or PCR purification column prior to electrotransformation. The desalted De-Cre reaction mix should not exceed 0.1 volumes of the electrocompetent cell suspension.

The cell/DNA mixture can be immediately used for electrotransformation without prior incubation on ice.
5. Add up to $400 \mu$ l of LB media (or SOC media) per $100 \mu$ l of cell/DNA suspension immediately after the transformation (heat shock or electroporation).
6. Incubate the suspension in a $37^{\circ} \mathrm{C}$ shaking incubator (recovery).

For recovery of partially deconstructed double/triple fusions, incubate the suspension in a $37^{\circ} \mathrm{C}$ shaking incubator for 1 to 2 hours.

For recovery of individual educts, incubate the suspension in a $37^{\circ} \mathrm{C}$ shaking incubator overnight or for at least 4 hours.
7. Plate out the recovered cell suspension on agar containing the desired (combination of) antibiotic(s). Incubate at $37^{\circ} \mathrm{C}$ overnight.
8. Colonies after overnight incubation can be verified directly by restriction digestion at this stage (refer to steps 12-16).
This is especially recommended in cases where only a single educt or partially deconstructed multifusion plasmid is desired.

For further selection by single antibiotic challenge on a 96 well microtiter plate, continue with step 9.
Several different single educts/partially deconstructed multifusion plasmids can be processed and selected in parallel on one 96 well microtiter plate.
9. For 96 well analysis, inoculate four colonies each from agar plates containing a defined set of antibiotics into approx. $500 \mu \mathrm{LB}$ medium without antibiotics. Incubate the cell cultures in a $37^{\circ} \mathrm{C}$ shaking incubator for 1-2 hours.
10. While incubating the colonies, fill a 96 well microtiter plate with $150 \mu$ l antibiotic-containing LB medium or dye (positional marker) in the corresponding wells.

Refer to Figures 9 and 10 for the arrangement of the solutions in the wells, which are used for parallel selection of single educts or partially deconstructed multifusion plasmids. The concept is that every cell suspension from a single colony needs to be challenged by all four antibiotics separately for unambiguous interpretation.
11. Add $1 \mu$ laliquots from the pre-incubated cell cultures (Step 9) into the corresponding wells. Incubate the 96 well microtiter plate in a $37^{\circ} \mathrm{C}$ shaking incubator overnight at $180-200 \mathrm{rpm}$.
Recommended: use parafilm to wrap the plate to prevent desiccation.
The remainder of the pre-incubated cell cultures can be kept at $4^{\circ} \mathrm{C}$ in a refrigerator for further inoculations if necessary.
12. Select transformants containing desired single educts or partially deconstructed multifusion plasmids according to the combination of dense (growth) and clear (no growth) cell cultures from each colony. Inoculate $10-20 \mu \mathrm{l}$ from the cell cultures into 10 ml LB media with corresponding antibiotic(s). Incubate in a $37^{\circ} \mathrm{C}$ shaking incubator overnight.
13. The next day, centrifuge the overnight cell cultures at 4000 g for $5-10$ minutes. Purify plasmid from cell pellets with common plasmid miniprep kits, according to manufacturers' protocols.
14. Determine the concentrations of purified plasmid solutions by using UV absorption spectroscopy (e.g. NanoDrop ${ }^{\text {TM }} 1000$ ).
15. Digest 0.5-1 $\mu \mathrm{g}$ of the purified plasmid solution in a $20 \mu \mathrm{l}$ restriction digestion (with 5-10 units endonuclease). Incubate under recommended reaction condition for approx. 2 hours.
16. Use 5-10 $\mu$ l of the digestion for analytical agarose gel (0.8-1.2\%) electrophoresis. Verify plasmid integrity by comparing the de facto restriction pattern to the in silico predicted restriction pattern (e.g. by using VectorNTI, Invitrogen, or any other similar program).
17. Optional: Occasionally, a deconstruction reaction is not complete but yields partially deconstructed fusions which still retain entities to be eliminated. In this case, we recommend to pick these partially deconstructed fusions containing and perform a second round of Cre deconstruction reaction (repeat steps 1-8) by using this construct as starting material.

## E. Appendix

## E.1. Preparing bacterial stock from agar stabs

We recommend that you prepare your personal bacterial stock from the agar stabs you received in the kit or transform your laboratory strain of choice with the vectors (please note that for the donor vectors this needs to be a pir+ strain). This is advisable since agar stabs only have a limited shelf life.

To generate your bacterial stock for long-term storage, streak bacteria from the agar stab onto an appropriate selective plate (refer to the vector maps for acceptor and donor vectors) or plates without antibiotics (pirHC and pirLC strains; we recommend to test these strains against a panel of antibiotics to be on the safe side; no growth of colonies should be observed under conditions of antibiotic selection). Incubate the plates over night at $37^{\circ} \mathrm{C}$ and then proceed to prepare stocks from individual colonies for long-term storage according to your protocol of choice (glycerol, DMSO, etc.), as described, for example, in Inoue et al. (1990), Molecular Cloning (Sambrook and Russell, 2000), Current Protocols in Molecular Biology (Ausubel et al., 1994), etc.

## E.2. MultiMam ${ }^{\text {™ }}$ Transient vectors: maps, sequences, MCS, restriction

Note: All acceptor and donor vector sequences can be provided in electronic format. These sequences contain all relevant information such as unique restriction sites, oris, resistance markers, etc. that is also shown in the circle maps. Request your set of vector files and accompanying files from Geneva Biotech at contact@geneva-biotech.com.

Acceptor and donor vectors are presented as circle maps and, in addition, the multiple cloning site (MCS) of each vector is shown featuring relevant unique restriction sites. Moreover, you will find, for the purposes of designing a restriction strategy, a non-exhaustive list of restriction endonucleases that cut once, twice or not at all. Additional restriction sites can be identified with any sequence analysis software, e.g. VectorNTI, ApE, etc. or by using online tools such as WebCutter 2.0 (http://rna.lundberg.gu.se/cutter2) or the NEB cutter V2.0 (http://tools.neb.com/NEBcutter2/).

## E.2.1 Acceptor vectors

## E.2.1.1 pACEMam1: 3443 bp



Multiple Cloning Site (promoter to terminator)
BamH Rsrif Bsshil EcoRI StuI SalI

GGCTAGTGGATCCCGGTCCGAAGCGCGCGGAATTCAAAGGCCTACGTCGACGAGCTCACTTGT
NotI BstBI XbaI PstI HindIII

CGCGGCCGCTTTCGAATCTAGAGCCTGCAGTCTCGACAAGCTTGTCGAGAAGTACTAGAGGA
Enzymes that cut pACEMam1 once (not exhaustive)

| 1 | Age I | 420 | AlwNI | 520 | ApaLI | 3254 | Avrll |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| 2801 | BamHI, Bstl | 3231 | BlpI | 114 | BspHI | 2867 | BstBI |
| 2816 | BssHII | 3266 | BstXI | 3260 | BstZ17I | 2074 | Bsu36I |
| 2101 | Clal | 1979 | DrallI | 2823 | EcoRI | 1224 | EcoRV |
| 2894 | HindIII | 3020 | Hpal | 3009 | Mfel | 2128 | Mlul |
| 1109 | Mscl | 2510 | Ncol | 2383 | Ndel | 2859 | Notl |
| 2087 | Pmel | 2881 | Pstl | 2103 | Pvul | 2807 | RsrlI |
| 1166 | Sacll | 2839 | Sall | 2907 | Scal | 2490 | SnaBI |
| 2833 | Stul | 2873 | Xbal | 1629 |  |  |  |

Bold type: restriction enzymes cutting in the MCS
Enzymes that cut pACEMam1 twice (not exhaustive)

| 2839,3260 | Accl | 2156,2761 | Asel / Vspl | 2714,2845 | Banll / Sacl |
| :--- | :--- | :--- | :--- | :--- | :--- |
| 949,1418 | BgllI | 1169,2859 | Eagl | 834,1647 | Pcil |
| 2095,2149 | Spel |  |  |  |  |

## Enzymes that do not cut pACEMam1 (not exhaustive)

| Acc65I | Aflll | Apal | Ascl | Bbsl | BsaBI |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Drall | EcoNI | Fsel | Kasl | Kpnl | Nael |
| Narl | Nhel | Nrul | Nsil | Pacl | Pfol |
| Pvull | Sbfl | Sfil | Sfol | Smal/Xmal | Sphl |
| Srfl | Sspl | Xcml | Xhol | Xmnl |  |

## Sequence

## 5'-

accggttgacttgggtcaactgtcagaccaagtttactcatatatactttagattgatttaaaact tcatttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatccctta acgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcc tttttttctgcgcgtaatctgctgcttgcaaacaaaaaaccaccgctaccagcggtggtttgttt gccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaa tactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacata cctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggtt ggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacaca gcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgc cacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcg cacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctg acttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgc ggcctttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccc tgattgacttgggtcgctcttcctgtggatgcgcagatgccctgcgtaagcgggtgtgggcggaca ataaagtcttaaactgaacaaaatagatctaaactatgacaataaagtcttaaactagacagaata gttgtaaactgaaatcagtccagttatgctgtgaaaaagcatactggacttttgttatggctaaag caaactcttcattttctgaagtgcaaattgcccgtcgtattaaagaggggcgtggccaagggcatg taaagactatattcgcggcgttgtgacaatttaccgaacaactccgcggccgggaagccgatctcg gcttgaacgaattgttaggtggcggtacttgggtcgatatcaaagtgcatcacttcttcccgtatg cccaactttgtatagagagccactgcgggatcgtcaccgtaatctgcttgcacgtagatcacataa gcaccaagcgcgttggcctcatgcttgaggagattgatgagcgcggtggcaatgccctgcctccgg tgctcgccggagactgcgagatcatagatatagatctcactacgcggctgctcaaacttgggcaga acgtaagccgcgagagcgccaacaaccgcttcttggtcgaaggcagcaagcgcgatgaatgtctta ctacggagcaagttcccgaggtaatcggagtccggctgatgttgggagtaggtggctacgtctccg aactcacgaccgaaaagatcaagagcagcccgcatggatttgacttggtcagggccgagcctacat gtgcgaatgatgcccatacttgagccacctaactttgttttagggcgactgccctgctgcgtaaca tcgttgctgctgcgtaacatcgttgctgctccataacatcaaacatcgacccacggcgtaacgcgc ttgctgcttggatgcccgaggcatagactgtacaaaaaacagtcataacaagccatgaaaaccgc cactgcgccgttaccaccgctgcgttcggtcaaggttctggaccagttgcgtgagcgcatacgcta cttgcattacagtttacgaaccgaacaggcttatgtcaactgggttcgtgccttcatccgtttcca cggtgtgcgtcacccggcaaccttgggcagcagcgaagtcgccataacttcgtatagcatacatta tacgaagttatctgtaactataacggtcctaaggtagcgagtttaaacactagtatcgatcgcgat gtacgggccagatatacgcgttgacattgattattgactagttattaatagtaatcaattacgggg tcattagttcatagcccatatatggagttccgcgttacataacttacggtaaatggcccgcctggc tgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaata gggactttccattgacgtcaatgggtggactatttacggtaaactgcccacttggcagtacatcaa gtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattat gcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctatt accatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactcacggggattt ccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttcca aaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctat ataagcagagctctctggctaactagagaacccactgcttactggcttatcgaaattaatacgact cactatagggagacccaagctggctagtggatcccggtccgaagcgcgcggaattcaaaggcctac
gtcgacgagctcacttgtcgcggccgctttcgaatctagagcctgcagtctcgacaagcttgtcga gaagtactagaggatcataatcagccataccacatttgtagaggttttacttgctttaaaaaacct cccacacctccccctgaacctgaaacataaaatgaatgcaattgttgttgttaacttgtttattgc agcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatttttttcact gcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctggatctgatcactgct tgagcctagaagatccggctgctaacaaagcccgaaaggaagctgagttggctgctgccaccgctg agcaataactatcataacccctagggtatacccatctaattggaaccagataagtgaaatctagtt ccaaactattttgtcatttttaattttcgtattagcttacgacgctacacccagttcccatctatt ttgtcactcttccctaaataatccttaaaactccatttccacccctcccagttcccaactatttt gtccgcccaca -3'

## E.2.1.2 pACEMam2: 4618 bp



Multiple Cloning Site (promoter to terminator)

BbsI XmaI
XhoI Ncol NheI PvuII
GCGGCCGTCTCAGGCCACCGAAGACTTGATCACCCGGGATCTCGAGCCATGGTGCTAGCAGCT
KpnI
NsiI SphI Acc65I
GATGCATAGCATGCGGTACCTAA

Enzymes that cut pACEMam2 once (not exhaustive)

| 2630 | Acc65I | 1 | Age I | 420 | AlwNI | 3573 | Apal |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| 520 | ApaLI | 4408 | Asel, Vspl | 2688 | Bbsl | 3278 | Blpl |
| 4441 | BstXI | 4417 | BstZ17I | 1224 | EcoRV | 888 | Fspl |
| $\mathbf{2 6 3 0}$ | Kpnl | 1109 | Mscl | $\mathbf{2 6 6 1}$ | Ncol | $\mathbf{2 6 5 4}$ | Nhel |
| $\mathbf{2 6 4 3}$ | Nsil | 2339 | PflMI | 2087 | Pmel | $\mathbf{2 6 4 9}$ | Pvull |
| $\mathbf{2 6 7 5}$ | Smal, Xmal | 4076 | SnaBI | 4421 | Spel | $\mathbf{2 6 3 6}$ | Sphl |
| $\mathbf{3 1 2 0}$ | Sse232I | $\mathbf{2 6 6 7}$ | Xhol, Scil |  |  |  |  |

Bold type: restriction enzymes cutting in the MCS

Enzymes that cut pACEMam2 twice (not exhaustive)

| 3478,3640 | Afel | 114,2509 | BspHI | 2967,3010 | Bbel, Kasl, Narl, Sfol |
| :--- | :--- | :--- | :--- | :--- | :--- |
| 2964,3121 | Nael | 1166,3405 | Sacll |  |  |

Enzymes that do not cut pACEMam2 (not exhaustive)

| AfIII | Ascl | Avrll | BamHI | Bcll | BsaBI |
| :--- | :--- | :--- | :--- | :--- | :--- |
| BspEl | BstBI | Clal | EcoNI | EcoRI | Fsel |


| HindlII | Hpal | Mfel | Mlul | Notl | Nrul |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Pacl | Pstl | Pvul | Rsrll | Sacl | Sall |
| Sbfl | Scal | Sfil | Sgfl | Srfl | Sspl |
| Stul | Xbal | Xcml | Xmnl |  |  |

## Sequence

5' -
accggttgacttgggtcaactgtcagaccaagtttactcatatatactttagattgatttaaaact tcatttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatccctta acgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcc tttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgttt gccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaa tactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacata cctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggtt ggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacggggggttcgtgcacaca gcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgc cacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcg cacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctg acttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgc ggcctttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccc tgattgacttgggtcgctcttcctgtggatgcgcagatgccctgcgtaagcgggtgtgggcggaca ataaagtcttaaactgaacaaaatagatctaaactatgacaataaagtcttaaactagacagaata gttgtaaactgaaatcagtccagttatgctgtgaaaaagcatactggacttttgttatggctaaag caaactcttcattttctgaagtgcaaattgcccgtcgtattaaagaggggcgtggccaagggcatg taaagactatattcgcggcgttgtgacaatttaccgaacaactccgcggccgggaagccgatctcg gcttgaacgaattgttaggtggcggtacttgggtcgatatcaaagtgcatcacttcttcccgtatg cccaactttgtatagagagccactgcgggatcgtcaccgtaatctgcttgcacgtagatcacataa gcaccaagcgcgttggcctcatgcttgaggagattgatgagcgcggtggcaatgccctgcctccgg tgctcgccggagactgcgagatcatagatatagatctcactacgcggctgctcaaacttgggcaga acgtaagccgcgagagcgccaacaaccgcttcttggtcgaaggcagcaagcgcgatgaatgtctta ctacggagcaagttcccgaggtaatcggagtccggctgatgttgggagtaggtggctacgtctccg aactcacgaccgaaaagatcaagagcagcccgcatggatttgacttggtcagggccgagcctacat gtgcgaatgatgcccatacttgagccacctaactttgttttagggcgactgccctgctgcgtaaca tcgttgctgctgcgtaacatcgttgctgctccataacatcaaacatcgacccacggcgtaacgcgc ttgctgcttggatgcccgaggcatagactgtacaaaaaaacagtcataacaagccatgaaaaccgc cactgcgccgttaccaccgctgcgttcggtcaaggttctggaccagttgcgtgagcgcatacgcta cttgcattacagtttacgaaccgaacaggcttatgtcaactgggttcgtgccttcatccgtttcca cggtgtgcgtcacccggcaaccttgggcagcagcgaagtcgccataacttcgtatagcatacatta tacgaagttatctgtaactataacggtcctaaggtagcgagtttaaacgtcgagggatcttcataa gagaagagggacagctatgactgggagtagtcaggagaggaggaaaaatctggctagtaaaacatg taaggaaaattttagggatgttaaagaaaaaaataacacaaaacaaaatataaaaaaatctaacc tcaagtcaaggcttttctatggaataaggaatggacagcagggggctgtttcatatactgatgacc tctttatagccacctttgttcatggcagccagcatatggcatatgttgccaaactctaaaccaaat actcattctgatgttttaaatgatttgccctcccatatgtccttccgagtgagagacacaaaaaat tccaacacactattgcaatgaaaataaatttcctttattagccagaagtcagatgctcaaggggct tcatgatgtccccataatttttggcagagggaaaaagatctcagtggtatttgtgagccagggcat tagccacaccagccaccaccttctgataggcagcctgcacctgaggagtgaattaggtaccgcatg ctatgcatcagctgctagcaccatggctcgagatcccgggtgatcaagtcttcggtggcctgagac ggccgcaattctttgccaaaatgatgagacagcacaacaaccagcacgttgcccaggagctgtagg aaaaagaagaaggcatgaacatggttagcagaggctctagcagccgccggtcacacgccagaagcc gaaccccgccctgccccgtcccccccgaaggcagccgtccccctgcggcagccccgaggctggaga tggagaaggggacggcggcgcggcgacgcacgaaggccctccccgcccatttccttcctgccggcg ccgcaccgcttcgcccgcgcccgctagagggggtgcggcggcgcctcccagatttcggctccgcca
gatttgggacaaaggaagtccctgcgccctctcgcacgattaccataaaaggcaatggctgcggct cgccgcgcctcgacagccgccggcgctccggggccgccgcgcccctcccccgagccctccccggcc cgaggcggccccgccccgcccggcacccccacctgccgccaccccccgcccggcacggcgagcccc gcgccacgccccgcacggagccccgcacccgaagccgggccgtgctcagcaactcggggagggggg tgcaggggggggttacagcccgaccgccgcgcccacaccccctgctcacccccccacgcacacacc ccgcacgcagcctttgttcccctcgcagcccccccgcaccgcggggcaccgcccccggccgcgctc ccctcgcgcacacgcggagcgcacaaagccccgcgccgcgcccgcagcgctcacagccgccgggca gcgcgggccgcacgcggcgctccccacgcacacacacacgcacgcaccccccgagccgctcccccc cgcacaaagggccctcccggagccctttaaggctttcacgcagccacagaaaagaaacgagccgtc attaaaccaagcgctaattacagcccggaggagaagggccgtcccgcccgctcacctgtgggagta acgcggtcagtcagagccggggcgggcggcgcgaggcggcgcggagcggggcacggggcgaaggca acgcagcgactcccgcccgccgcgcgcttcgctttttatagggccgccgccgccgccgcctcgcca taaaaggaaactttcggagcgcgccgctctgattggctgccgccgcacctctccgcctcgccccgc cccgcccctcgccccgccccgccccgcctggcgcgcgccccccccccccccgcccccatcgctgca caaaataattaaaaaataaataaatacaaaattgggggtggggaggggggggagatggggagagtg aagcagaacgtggggctcacctcgacccatgagtaatagcgatgactaatacgtagatgtactgcc aagtaggaaagtcccataaggtcatgtactgggcataatgccaggcgggccatttaccgtcattga cgtcaatagggggcgtacttggcatatgatacacttgatgtactgccaagtgggcagtttaccgta aatagtccacccattgacgtcaatggaaagtccctattggcgttactatgggaacatacgtcatta ttgacgtcaatgggcgggggtcgttgggcggtcagccaggcgggccatttaccgtaagttatgtaa cgcggaactccatatatgggctatgaactaatgaccccgtaattgattactattaataacgtatac tagtatcgtagtatcgatccatctaattggaaccagataagtgaaatctagttccaaactattttg tcatttttaattttcgtattagcttacgacgctacacccagttcccatctattttgtcactcttcc ctaaataatccttaaaaactccatttccacccctcccagttcccaactattttgtccgcccaca $3^{\prime}$

## E.2.2 Donor vectors

## E.2.2.1 pMDC: 2889 bp



Multiple Cloning Site (promoter to terminator)

|  |  | BamHI |  | BsshII |  | StuI | SalI |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| AGACCCAAGCTGGCTAGTGGATCCCGGTCCGAAGCGCGCGGAATTCAAAGGCCTACGTCGAC |  |  |  |  |  |  |  |
| SacI |  | XbaI |  |  | PstI |  |  |
| GAGCTCACTAGTCGCGGCCGCTTTCGAATCTAGAGCCTGCAGTCTCGACAA |  |  |  |  |  |  |  |
| Enzymes that cut pMDC once (not exhaustive) |  |  |  |  |  |  |  |
| 1302 | AccIII, BspEI | 54 | Acc65I | 693 | Age I | 1723 | Avrll |
| 2176 | BamHI | 1682 | BgIII | 2161 | BssHII | 5 | BstXI |
| 643 | Bsu36I | 2877 | Clal | 1957 | Hpal | 54 | Kpnl |
| 1968 | Mfel | 2849 | Mlul | 1033 | Mscl | 2871 | Nrul |
| 2096 | Pstl | 2169 | RsrlI | 58 | Sacll | 2138 | Sall |
| 988 | Sspl | 2146 | Stul | 2104 | Xbal |  |  |
| Bold type: restriction enzymes cutting in the MCS |  |  |  |  |  |  |  |

## Enzymes that cut pMDC twice (not exhaustive)

| 768,2110 | BstBI | 61,2118 | BstZI, Eagl | 1298,2154 | EcoRI |
| :--- | :--- | :--- | :--- | :--- | :--- |
| 349,2083 | HindIII | 997,2467 | Ncol | 60,2117 | NotI |
| 1634,1668 | PflMI | 316,1937 | Psil | 573,1400 | Pvull |
| 2132,2263 | Sacl | 883,2070 | Scal | 107,2489 | SnaBI |

Enzymes that do not cut pMDC (not exhaustive)

| Afel | AfllI | AlwNI | Apal | Ascl | Bbel |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Bbsl | Bcll | BspHI | BstZ17I | Drall | EcoNI |
| EcoRV | Fsel | Fspl | Kasl | Nael | Narl |


| Nhel | Nsil | Pacl | Pmel | Pvul | Sbfl |
| :--- | :--- | :--- | :--- | :--- | :--- |
| Scil | Sfil | Sgfl | Smal | Sphl | Xcml |
| Xhol | Xmal | Xmnl |  |  |  |

## Sequence

## 5'-

aaacccatgtgcctggcagataacttcgtataatgtatgctatacgaagttatggtaccgcggccgcgtagaggat ctgttgatcagcagttcaacctgttgatagtacgtactaagctctcatgtttcacgtactaagctctcatgtttaa cgtactaagctctcatgtttaacgaactaaaccctcatggctaacgtactaagctctcatggctaacgtactaagc tctcatgtttcacgtactaagctctcatgtttgaacaataaaattaatataaatcagcaacttaaatagcctctaa ggttttaagttttataagaaaaaaagaatatataaggcttttaaagcttttaaggtttaacggttgtggacaaca agccagggatgtaacgcactgagaagcccttagagcctctcaaagcaattttgagtgacacaggaacacttaacgg ctgacatgggaattagcttcacgctgccgcaagcactcagggcgcaagggctgctaaaggaagcggaacacgtaga aagccagtccgcagaaacggtgctgaccccggatgaatgtcagctgggaggcagaataaatgatcatatcgtcaat tattacctccacggggagagcctgagcaaactggcctcaggcatttgagaagcacacggtcacactgcttccggta gtcaataaaccggtaaaccagcaatagacataagcggctatttaacgaccctgccctgaaccgacgaccgggtcga atttgctttcgaatttctgccattcatccgcttattatcacttattcaggcgtagcaaccaggcgtttaagggcac caataactgccttaaaaaattacgccccgccctgccactcatcgcagtactgttgtaattcattaagcattctgc cgacatggaagccatcacaaacggcatgatgaacctgaatcgccagcggcatcagcaccttgtcgccttgcgtata atatttgcccatggtgaaaacgggggcgaagaagttgtccatattggccacgtttaaatcaaaactggtgaaactc acccagggattggctgagacgaaaaacatattctcaataaaccctttagggaaataggccaggttttcaccgtaac acgccacatcttgcgaatatatgtgtagaaactgccggaaatcgtcgtggtattcactccagagcgatgaaaacgt ttcagtttgctcatggaaaacggtgtaacaagggtgaacactatcccatatcaccagctcaccgtctttcattgcc atacggaattccggatgagcattcatcaggcgggcaagaatgtgaataaaggccggataaaacttgtgcttatttt tctttacggtctttaaaaaggccgtaatatccagctgaacggtctggttataggtacattgagcaactgactgaaa tgcctcaaaatgttctttacgatgccattgggatatatcaacggtggtatatccagtgatttttttctccatttta gcttccttagctcctgaaaatctcgataactcaaaaaatacgcccggtagtgatcttatttcattatggtgaaagt tggaccctcttacgtgccgatcaacgtctcattttcgccaaaagttggcccagatctatgtcgggtgcggagaaag aggtaatgaaatggcacctaggggttatgatagttattgctcagcggtggcagcagccaactcagcttcctttcgg gctttgttagcagccggatcttctaggctcaagcagtgatcagatccagacatgataagatacattgatgagtttg gacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaac cattataagctgcaataaacaagttaacaacaacaattgcattcattttatgtttcaggttcagggggaggtgtgg gaggttttttaaagcaagtaaaacctctacaaatgtggtatggctgattatgatcctctagtacttctcgacaagc ttgtcgagactgcaggctctagattcgaaagcggccgcgactagtgagctcgtcgacgtaggcctttgaattccgc gcgcttcggaccgggatccactagccagcttgggtctccctatagtgagtcgtattaatttcgataagccagtaag cagtgggttctctagttagccagagagctctgcttatatagacctcccaccgtacacgcctaccgcccatttgcgt caatggggcggagttgttacgacattttggaaagtcccgttgattttggtgccaaaacaaactcccattgacgtca atggggtggagacttggaaatccccgtgagtcaaaccgctatccacgcccattgatgtactgccaaaaccgcatca ccatggtaatagcgatgactaatacgtagatgtactgccaagtaggaaagtcccataaggtcatgtactgggcata atgccaggcgggccatttaccgtcattgacgtcaatagggggcgtacttggcatatgatacacttgatgtactgcc aagtgggcagtttaccgtaaatactccacccattgacgtcaatggaaagtccctattggcgttactatgggaacat acgtcattattgacgtcaatgggcgggggtcgttgggcggtcagccaggcgggccatttaccgtaagttatgtaac gcggaactccatatatgggctatgaactaatgaccccgtaattgattactattaataactagtcaataatcaatgt caacgcgtatatctggcccgtacatcgcgaatcgatactagta
$-3^{\prime}$

## E.2.2.2 pMDK: 4130 bp



Multiple Cloning Site (promoter to terminator)

BbsI XhoI NcoI NheI
GCGGCCGTCTCAGGCCACCGAAGACTTGATCACCCGGGATCTCGAGCCATGGTGCTAGCAGCT
Acc65I
NsiI
KpnI
GATGCATAGCATGCGGTACCTAA

Enzymes that cut pMDK once (not exhaustive)

| $\mathbf{2 9 0 4}$ | Acc65I, KpnI | 3967 | AlwNI | 1961 | Apal | 1100 | AvrlI |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| 3025 | BspHI | 899 | BstBI | 3453 | BstXI | 1117 | BstZ17I |
| 2919 | Bsu36I | 1107 | Clal | 318 | Fspl | 3746 | HindIII |
| 298 | Mscl | $\mathbf{2 8 7 3}$ | Ncol | $\mathbf{2 8 8 0}$ | Nhel | $\mathbf{2 8 9 1}$ | Nsil |
| 3190 | PfIMI | 3445 | Pmel | 267 | Pstl | $\mathbf{7 3 3}$ | RsrlI |
| 2129 | Sacll | 1113 | Spel | 27 | Xcml | $\mathbf{2 8 6 7}$ | Xhol |

Bold type: restriction enzymes cutting in the MCS

Enzymes that cut pMDK twice (not exhaustive)

| 1894,2056 | Afel | 1126,3668 | Asel | 54,2990 | BgIII |
| :--- | :--- | :--- | :--- | :--- | :--- |
| 993,1954 | Pfol | 1055,2859 | Smal, Xmal | 1458,3504 | SnaBI |
| 619,2898 | Sphl |  |  |  |  |

Enzymes that do not cut pMDK (not exhaustive)

| AccllI | Acll | Aflll | Agel | ApaLI | Ascl |
| :--- | :--- | :--- | :--- | :--- | :--- |
| BamHI | Bcll | BspEl | EcoNI | EcoRI | EcoRV |
| Fsel | Hpal | Mfel | Mlul | Notl | Nrul |
| Pacl | Pvul | Sacl | Sall | Sbfl | Scal |

## Sequence

$5^{\prime}-$
aagtaaactggatggctttcttgccgccaaggatctgatggcgcaggggatcaagatctgatcaag agacaggatgaggatcgtttcgcatgattgaacaagatggattgcacgcaggttctccggccgctt gggtggagaggctattcggctatgactgggcacaacagacaatcggctgctctgatgccgccgtgt tccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatg aactgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgc tcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctcc tgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcata cgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactc ggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccg aactgttcgccaggctcaaggcgcgcatgcccgacggcgaggatctcgtcgtgacacatggcgatg cctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggctgg gtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcg aatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttct atcgccttcttgacgagttcttctgagcgggactctggggttcgaaatgaccgaccaagcgacgcc caacctgccatcacgagatttcgattccaccgccgccttctatgaaaggttgggcttcggaatcgt tttccgggacgccggctggatgatcctccagcgcggggatctcatgctggagttcttcgcccaccc cgggatctatgtcgggtgcggagaaagaggtaatgaaatggcacctaggtatcgatactagtatac gttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttacat aacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatga cgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggactatttacggt aaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatg acggtaaatggcccgcctggcattatgcccagtacatgaccttatgggactttcctacttggcagt acatctacgtattagtcatcgctattactcatgggtcgaggtgagccccacgttctgcttcactct cсссаtсtссссссссtссссасссссаattttgtatttatttattttttaattattttgtgcagc gatgggggcogggggggggggggcocgcoccaggcggggcggggcggggcgaggggcggggcgggg cgaggcggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttccttttatggcga ggcggcggcggcggcggccctataaaaagcgaagcgcgcggcgggcgggagtcgctgcgttgcctt cgcccegtgccecgctccgcgccgcctcgcgccgcccgccecggctctgactgaccgcgttactcc cacaggtgagcgggcgggacggcccttctcctccgggctgtaattagcgcttggtttaatgacggc tcgtttcttttctgtggctgcgtgaaagccttaaagggctccgggagggccctttgtgcggggggg agcggctcggggggtgcgtgcgtgtgtgtgtgcgtggggagcgccgcgtgcggcccgcgctgcccg gcggctgtgagcgctgcgggcgcggcgcggggctttgtgcgctccgcgtgtgcgcgaggggagcgc ggccgggggcggtgccccgcggtgcgggggggctgcgaggggaacaaaggctgcgtgcggggtgtg tgcgtgggggggtgagcagggggtgtgggcgcggcggtcgggctgtaacccccccctgcacccccc tccccgagttgctgagcacggcccggcttcgggtgcggggctccgtgcggggcgtggcgcggggct cgccotgccoggcggggggtggcggcaggtgggggtgccoggcggggcggggccgcctcgggccgg ggagggctcgggggaggggcgcggcggccccggagcgccggcggctgtcgaggcgcggcgagccgc agccattgccttttatggtaatcgtgcgagagggcgcagggacttcctttgtcccaaatctggcgg agccgaaatctgggaggcgccgccgcaccccctctagcgggcgcgggcgaagcggtgcggcgccgg caggaaggaaatgggcggggagggccttcgtgcgtcgccgcgccgccgtccccttctccatctcca gcctcggggctgccgcagggggacggctgccttcgggggggacggggcagggcggggttcggcttc tggcgtgtgaccggcggctgctagagcctctgctaaccatgttcatgccttcttctttttcctaca gctcctgggcaacgtgctggttgttgtgctgtctcatcattttggcaaagaattgcggccgtctca ggccaccgaagacttgatcacccgggatctcgagccatggtgctagcagctgatgcatagcatgcg gtacctaattcactcctcaggtgcaggctgcctatcagaaggtggtggctggtgtggctaatgccc tggctcacaaataccactgagatctttttccctctgccaaaaattatggggacatcatgaagcccc ttgagcatctgacttctggctaataaaggaaatttattttcattgcaatagtgtgttggaattttt tgtgtctctcactcggaaggacatatgggagggcaaatcatttaaaacatcagaatgagtatttgg

## - 37 -

tttagagtttggcaacatatgccatatgctggctgccatgaacaaaggtggctataaagaggtcat cagtatatgaaacagccccctgctgtccattccttattccatagaaaagccttgacttgaggttag attttttttatattttgttttgtgttatttttttctttaacatccctaaaattttccttacatgtt ttactagccagatttttcctcctctcctgactactcccagtcatagctgtccctcttctcttatga agatccctcgacgtttaaacccatgtgcctggcagataacttcgtataatgtatgctatacgaagt tatggtacgtactaagctctcatgtttcacgtactaagctctcatgtttaacgtactaagctctca tgtttaacgaactaaaccctcatggctaacgtactaagctctcatggctaacgtactaagctctca tgtttcacgtactaagctctcatgtttgaacaataaaattaatataaatcagcaacttaaatagcc tctaaggttttaagttttataagaaaaaaaagaatatataaggcttttaaagcttttaaggtttaa cggttgtggacaacaagccagggatgtaacgcactgagaagcccttagagcctctcaaagcaattt tcagtgacacaggaacacttaacggctgacagaattagcttcacgctgccgcaagcactcagggcg caagggctgctaaaggaagcggaacacgtagaaagccagtccgcagaaacggtgctgaccccggat gaatgtcagctactgggctatctggacaagggaaaacgcaagcgcaaagagaaagcaggtagcttg cagtgggcttacatggcgatagctagactgggcggttttatggacagcaagcgaaccggaattgcc agctggggcgccctctggtaaggttgggaagccctgca-3'

## E.2.2.3 pMDS: 4088 bp



Multiple Cloning Site (promoter to terminator)

BbsI XmaI SciI |  | SmaI NheI NcoI NheI |
| :--- | :--- |

GCGGCCGTCTCAGGCCACCGAAGACTTGATCACCCGGGATCTCGAGCCATGGTGCTAGCAGCT
Acc65I
NsiI SphI KpnI
GATGCATAGCATGCGGTACCTAA

| Enzymes that cut pMDS once (not exhaustive) |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1804 | Acc651, Kpnl | 2988 | Agel | 861 | Apal | 3495 | ApaLI |
| 4080 | Avrlı | 1746 | Bbsl | 1890 | BgIII | 3380 | Bspell |
| 2353 | BstXI | 17 | BstZ17I | 4087 | Clal | 2646 | HindIII |
| 1773 | Ncol | 1780 | Nhel | 1791 | Nsil | 2090 | PfIMI |
| 2345 | Pmel | 2613 | Psil | 3598 | Pvul | 1029 | Sacll |
| 1767 | Scil | 1759 | Smal, Xmal | 13 | Spel | 1798 | Sphl |
| 1312 | Sse2321 | 1767 | Xhol |  |  |  |  |

Bold type: restriction enzymes cutting in the MCS

| Enzymes that cut pMDS twice (not exhaustive) |  |  |  |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- |
| 794,956 | Afel, Vspl | 26,2568 | Asel | 1424,1467 | Bbel, Narl |
| 1925,3220 | BspHI | 1819,2938 | Bsu36I | 1012,1728 | Eagl |
| 1785,2868 | Pvull | 358,2404 | SnaBI |  |  |

## Enzymes that do not cut pMDS (not exhaustive)

| Acll | AlwNI | Ascl | BamHI | Bcll | BsaBI |
| :--- | :--- | :--- | :--- | :--- | :--- |
| BspEl | BstBI | EcoNI | EcoRI | EcoRV | Fsel |
| Fspl | Hpal | Mfel | Mlul | Mscl | Notl |
| Nrul | Pacl | Pmll | Pstl | Rsrll | Sacl |
| Sall | Sbfl | Scal | Sfil | Sgfl | Stul |
| Xbal | Xcml | Xmnl |  |  |  |

## Sequence

5' -
cgatactacgatactagtatacgttattaatagtaatcaattacggggtcattagttcatagccca tatatggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccc cgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgt caatgggtggactatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagt acgccccctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagtacatgacctta tgggactttcctacttggcagtacatctacgtattagtcatcgctattactcatgggtcgaggtga gccccacgttctgcttcactctccccatctcccccccctccccacccccaattttgtatttattta ttttttaattattttgtgcagcgatgggggcggggggggggggggcgcgcgccaggcggggcgggg cggggcgaggggcggggcggggcgaggcggagaggtgcggcggcagccaatcagagcggcgcgctc cgaaagtttccttttatggcgaggcggcggcggcggcggccctataaaaagcgaagcgcgcggcgg gcgggagtcgctgcgttgccttcgccccgtgccccgctccgcgccgcctcgcgccgcccgccccgg ctctgactgaccgcgttactcccacaggtgagcgggcgggacggccettctcctccgggctgtaat tagcgcttggtttaatgacggctcgtttcttttctgtggctgcgtgaaagccttaaagggctccgg gagggccctttgtgcgggggggagcggctcggggggtgcgtgcgtgtgtgtgtgcgtggggagcgc cgcgtgcggcccgcgctgcccggcggctgtgagcgctgcgggcgcggcgcggggctttgtgcgctc cgcgtgtgcgcgaggggagcgcggccgggggcggtgccccgcggtgcgggggggctgcgaggggaa caaaggctgcgtgcggggtgtgtgcgtgggggggtgagcagggggtgtgggcgcggcggtcgggct gtaacccccccctgcacccccctccccgagttgctgagcacggcccggcttcgggtgcggggctcc gtgcggggcgtggcgcggggctcgccgtgccgggcggggggtggcggcaggtgggggtgccgggcg gggcggggccgcctcgggccggggagggctcgggggaggggcgcggcggccccggagcgccggcgg ctgtcgaggcgcggcgagccgcagccattgccttttatggtaatcgtgcgagagggcgcagggact tcctttgtcccaaatctggcggagccgaaatctgggaggcgccgccgcaccccctctagcgggcgc gggcgaagcggtgcggcgccggcaggaaggaaatgggcggggagggccttcgtgcgtcgccgcgcc gccgtccccttctccatctccagcctcggggctgccgcagggggacggctgccttcgggggggacg gggcagggcggggttcggcttctggcgtgtgaccggcggctgctagagcctctgctaaccatgttc atgccttcttctttttcctacagctcctgggcaacgtgctggttgttgtgctgtctcatcattttg gcaaagaattgcggccgtctcaggccaccgaagacttgatcacccgggatctcgagccatggtgct agcagctgatgcatagcatgcggtacctaattcactcctcaggtgcaggctgcctatcagaaggtg gtggctggtgtggctaatgccctggctcacaaataccactgagatctttttccctctgccaaaaat tatggggacatcatgaagccccttgagcatctgacttctggctaataaaggaaatttattttcatt gcaatagtgtgttggaattttttgtgtctctcactcggaaggacatatgggagggcaaatcattta aaacatcagaatgagtatttggtttagagtttggcaacatatgccatatgctggctgccatgaaca aaggtggctataaagaggtcatcagtatatgaaacagccccctgctgtccattccttattccatag aaaagccttgacttgaggttagattttttttatattttgttttgtgttatttttttctttaacatc cctaaaattttccttacatgttttactagccagatttttcctcctctcctgactactcccagtcat agctgtccctcttctcttatgaagatccctcgacgtttaaacccatgtgcctggcagataacttcg tataatgtatgctatacgaagttatggtacgtactaagctctcatgtttcacgtactaagctctca tgtttaacgtactaagctctcatgtttaacgaactaaaccctcatggctaacgtactaagctctca tggctaacgtactaagctctcatgtttcacgtactaagctctcatgtttgaacaataaaattaata taaatcagcaacttaaatagcctctaaggttttaagttttataagaaaaaaaagaatatataaggc
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## NOTES:

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