Quantitative Models for Efficient Cloning of Different Vectors with Various Clone sites

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Abstract

We developed an efficient strategy, “Combinatorial Strategy”, for cloning different vectors with various clone sites. 1) Using originally existed clone sites from circular vectors to prepare the inserts, and if no appropriate sites available, performing SDM to create compatible sites, to achieve maximal correct digestion of the inserts. 2) Different vectors were digested with various restriction endonucleases, and then dephosphorylated with CIP. 3) Top10 competent cells were used for transformation to increase the transformant colonies. Our results showed that, when blunt sites, or a Xba I site was adopted for ligation, the percentages of positive clones were about 50%. Whereas, when different sites, including one blunt and another Pst I sites, Not I and Xho I sites, the percentages of positive clones were nearly 100%. Using this strategy, most vectors could be successfully cloned through “one ligation, one transformation, three to five minipreps”.

Key words: Quantitative models, Cloning vectors, Calf intestinal phosphatase (CIP) treatment, Site-directed mutagenesis (SDM), Combinatorial strategy, Top10, DNA recombination.

Introduction

The successful production and intracellular replication of recombinant DNA were first reported in 1972 and 1973[1, 2], and these techniques have significantly revolutionized biological sciences in the past decades. At the genome era, more and more gene sequences of a growing number of species become available, and many different vector systems have been developed by scientists. Therefore, there is an increasing need for efficient strategies to clone interested genes, precisely and efficiently, into various expression vectors according to specific aims. For example, to establish stable transgene cell lines, lentiviral vectors (LVs) might be a good choice, and to achieve conditional transgenesis and gene knockdown, drug-inducible systems were designed for these purposes[3-12].

Generally, the DNA recombination techniques were of low efficiency and randomness. To overcome the randomness of vector cloning with certainty, recently, we reported an efficient cloning method, "Combinatorial strategy", for lentiviral vectors using site-directed mutagenesis (SDM) to insert clone site BamH I[13]. SDM was first established at 1978 by Hutchison *et al*., and it is essential in gene functional studying, genetic engineering, protein engineering, and vector modifications[14-16]. Currently, the QuikChange™ SDM System developed by Stratagene is a commonly used kit for mutagenesis using plasmid double-stranded DNA as templates. The advantage of this strategy is that the products after mutagenesis are circular, double-stranded plasmid DNA. As results, theoretically, 100% of the linearized DNA fragments are with correct-cleaved ends. Therefore, maximal efficiencies of ligation could be achieved[13].

In our neuroscience lab focused on Parkinson disease, we need to clone several different kinds of expression vectors for α-synuclein (α-Syn) and related genes, such as Rab3A, GDI, β5, Plk2[17], etc., both wild type (WT) and mutants, to investigate their functional relations *in vitro* and *in vivo*. The aim of this study is to generalize our reported combinatorial strategy to efficiently construct different expression vectors with various clone

sites, such as blunt-end sites (Swa I, Pme I and EcoR V), one blunt-end and one-overhang-end sites (Pme I and Pst I), two different overhang-end sites (Not I and Xho I), and one overhang-end site (Xba I), etc.. Encouraged by our former report[13], with our optimized method, we successfully constructed 18 different vectors, mostly through "one ligation, one transformation, and a few minipreps", for each of them. The percentages of positive clones with blunt sites, different clone sites, and a Xba I site, were approximately 47% ±31% (n=13), 93% ±12% (n=3), and 36% ±10% (n=2), respectively. This study provided important quantitative models for efficient construction of different vectors, and laid the significant foundations for gene functional analyses.

Results

Pst I and Xba I insertion by SDM

SDM was performed by PCR, and the parental template DNA was digested with Dpn I. Different annealing temperatures were used for pcDNA4/β5WT/3'-Pst I, pcDNA4/β5T1A/3'-Pst I, pcDNA4/β5TW/5'-Xba I, pcDNA4/β5T1A/5'-Xba I, and pcDNA4/β5T1A/5'-Xba I/3'-Xba I insertions, representing approximately T_m^*-9 ℃, T_m + 3 ℃, and T_m^*-5 ℃ (**Table 1 and 2; Figure 2 and 3**), respectively. After transformation into Top10 or DH5α cells and identified by sequencing, our results showed that, the mutagenesis efficiencies were approximately 50% for pcDNA4/β5WT/3'-Pst I insertion, 33.3% for pcDNA4/β5T1A/3'-Pst I insertion, 10% for pcDNA4/β5T1A/5'-Xba I insertion, and 50% for pcDNA4/β5T1A/5'-Xba I/3'-Xba I insertion, respectively. In contrast, positive mutagenesis clones were not obtained for pcDNA4/β5WT/5'-Xba I insertion after sequencing 18 minipreps (**Table 1 and 2**). These data demonstrated that the majority of our designed mutagenesis was successfully achieved.

Efficient cloning of different vectors with various clone sites

Construction of LVs with blunt-end clone sites

A blunt-end clone site Swa I of pWPI/Neo LV was employed as clone site. To protect the vector self-circularization, we removed the 5'-phosphate groups of the vector DNA with calf intestinal phosphatase (CIP) treatment following Swa I digestion. This treatment could diminish the background of transformed colonies that carried empty plasmids. At the same time, the colony number would be significantly decreased after transformation[18-20]. Insert (α-Syn WT and A30P, A53T mutants; Rab3A WT and T36N, Q81L mutants; GDI WT and R218E, R240A mutants; β5 WT and T1A mutant; respectively) and vector were pooled together in 10μl ligation reactions with DNA concentrations around 18.9~42ng/μl, and insert to vector molar ratios about 1~3.4**:**1 (**Table 3, Figure 1**). To improve the transformation efficiencies, Top10 cells were used as hosts, and 2 to about 20 colonies were obtained after transformation. After identification by restriction endonulease digestion and sequencing, the percentages of positive clones with monomeric, correct-oriented inserts were ranging from 20% to 100%, respectively (**Table 3**). All the blunt-end cloning described above was achieved by performing one ligation and one transformation, except the cloning of pWPI/GDIWT/Neo. 15 colonies were resulted from the first ligation and transformation, and after identification with BamH I digestion, 9 clones were with opposite-directed inserts, and the other 6 clones were with unknown DNAs. Through the second ligation and transformation, positive clones of pWPI/GDIWT/Neo were obtained (**Table 3**).

To construct inducible LVs, pLenti CMV/TO Puro DEST was digested with EcoR V, and followed by CIP treatment. In 10μl ligation system, the molar ratios of insert β5 WT and T1A mutant to pLenti vector were 22:1 and 14.1:1, and the DNA amounts were about 18.9ng/μl and 14ng/μl, respectively (**Table 3, Figure 1**). After transformation into Top10 cells, 12 and 13 colonies were obtained, and the percentages of positive clones were 37.5% and 12.5%, respectively. Taken together, the percentages of inserted clones and positive clones with blunt-end ligations, were approximately 77% ±25%, and 47% ±31%, respectively (**Table 3, Figure 4**).

Construction of different vectors with various clone sites

pLVCT-tTR-KRAB was first digested with Pme I, then with Pst I, sequentially, and then treated with CIP. In 10μl ligation system, the molar ratios of inserts β5WT and T1A mutant to pLVCT vector were 3:1, and the DNA amounts were 17.6ng/μl, respectively (**Table 3, Figure 2**). After transformation into Top10 cells, about 100 and 300 colonies were obtained. After identification, all the recombinant clones were with correct directions, therefore, the percentages of inserted clones and positive clones were both 80% and 100%, respectively (Table 3).

Due to the failure to insert 5'-Xba I site for pcDNA4/β5WT (**Table 2**), pcDNA4/β5T1A/5'-Xba I/3'-Xba I was digested with Not I and Xho I, simultaneously, and followed by CIP treatment, the insert β5WT (digested with Not I and Xho I) was ligated into the vector pcDNA4/5'-Xba I/3'-Xba I to form pcDNA4/β5WT/5'-Xba I/3'-Xba I (**Figure 3**). The molar ratio of insert β5WT to pcDNA4/5'-Xba I/3'-Xba I vector was 1.9:1, and the DNA amount was 28.1ng/μl, respectively. After transformation into Top10 cells, about 500 colonies were obtained, the percentages of inserted clones and positive clones were both 100% (**Table 3**).

Construction of inducible pTet/β5WT andpTet/β5T1A vectors with a Xba I clone site

Previously, we reported the construction of pWPI/hPlk2 WT and mutant vectors with a BamH I site by inserting BamH I clone site at the 3'-ends of hPlk2[13]. The pTetO-HGMoPrP vector was digested with Xba I to cut off the insert HGMoPrP, followed by CIP treatment. β5WT and β5T1A inserts were cleaved from vectors pcDNA4/5'-Xba I/3'-Xba I, and ligated with pTet by Xba I clone site, to form inducible vector pTet/β5WT and pTet/β5T1A (**Figure 3**). In 10μl ligation system, the molar ratios of insert β5WT and T1A mutant to pTet vector were 2.1:1 and 1.5:1, and the DNA amounts were 21.9ng/μl and 19.7ng/μl,

respectively. After transformation into Top10 cells, about 1000 colonies of each were obtained. The percentages of inserted clones were 100% for both of them, and those of positive clones were 28.6% and 42.9%, respectively (**Table 3**).

Taken together, the percentages of inserted clones and positive clones with ligation of blunt-end sites (including Swa I, Pme I and EcoR V), different clone sites (EcoR V, Pme I and Pst I; Not I and Xho I), and a Xba I site **(Figure 1, 2 and 3)**, were approximately 77% ±25% and 47% ±31% (n=13), both 93% ±12% (n=3), and 100% ±0 and 36% ±10% (n=2) of identified clones, respectively. Among them, the efficiencies of blunt-end cloning and Xba I site cloning were not significantly different (47% ±31% vs 36% ±10%, *P>0.05*), these data were similar with our previous report with a BamH I clone site[13] (**Table 3**). In contrast, the efficiencies between blunt-end cloning and different site cloning (47% ±31% vs 93% ±12%), different site cloning and Xba I site cloning (93% ±12% vs 36% ±10%) were significantly different (*P<0.05*).

Discussion

Creation of compatible clone sites between vectors and inserts is the first step for successful cloning, and it is critical that the cutting-ends of DNA fragments are correctly generated after restriction digestion, particularly for large-size, low-efficiency cloning. Generally, LVs carry limited clone sites, and a unique clone site usually used for cloning, such as BamH I or EcoR V[7, 13, 21] etc. Therefore, it is necessary to create compatible clone sites between the vectors and inserts. SDM is a powerful tool to change DNA sequences at specific positions in genetic engineering, including insertion of clone sites[15, 16, 22]. According to the working format of the QuikChange™ SDM System, double-stranded plasmid DNA was used as templates, therefore, after Dpn I digestion and transformation, the mutagenesis products are circular double-stranded plasmid DNA (Stratagene). As a result, after restriction digestion, 100% of the purified linearized DNA fragments through Agarose Gel Electrophoresis were theoretically with correct ends. Whereas, the correct cutting ends could

not be confirmed with the method of incorporating of restriction sites into PCR primers[23, 24]. In this study, Pst I clone site at the 3'-ends of β5 WT and T1A mutant genes was inserted by SDM, to achieve the cloning with 5'-blunt end and 3'-Pst I end between pLVCT vector and β5 WT and T1A mutant inserts. Our data demonstrated that the clone efficiencies were significantly improved with this strategy. The percentages of positive clones with monomeric, correct-oriented inserts were 80% for pLVCT/β5WT, and 100% for pLVCT/β5T1A, respectively (**Table 3, Figure 2**). In addition, Xba I site was also inserted at both 5'- and 3'-ends of β5T1A mutant, and Tet-inducible expression vectors, pTet/β5WT and pTet/β5T1A, were constructed with a Xba I site between pTet vector and β5 WT and T1A mutant inserts. The percentages of positive clones were 28.6% and 42.9% (36% ±10%, n=2), respectively (**Table 3, Figure 3**). These data were consistent with our former report[13].

Ligation and transformation are complicated procedures, and many factors could affect their efficiencies, such as the dephosphorylation of the vectors, the concentrations and ratios of the vector and insert DNA, the amount of DNA used for transformation, and so on. Removing the 5'-phosphate residues from both termini of the vector DNA can efficiently minimize the re-circularization of vector DNA, and therefore, decrease the background with empty transformants. At the same time, the transformation colonies were significantly decreased, particularly for ligation with blunt ends, because the ligation efficiency of blunt ends was much lower than cohesive end ligation[25, 26]. Our data also demonstrated this phenomenon (**Table 3**). In our experiment, the transformant number of blunt-end ligations was from 2 to 20, whereas, the transformation colonies of the ligation with one blunt end and one cohesive end were about 100 and 300, respectively. In addition, about 500 colonies were obtained when Not I and Xho I sites were used for ligation, and about 1000 colonies were resulted from Xba I site ligation (**Table 3**). Furthermore, the ligation efficiencies were significantly affected by vector sizes. For instance, when pLVCT (12kb), pcDNA4 (5.1kb), and pTet (3.2kb) were used for ligation with β5 WT and T1A mutant inserts, more transformation colonies were obtained with the decrease of vector sizes (**Table 3**). To investigate the effects of the molar ratios of insert to vector on the transformant number and the percentages of positive clones, a large range of molar ratios, from 1.7 to 22:1, was used for ligation with blunt ends, there was no significant different between the number of obtained

transformants and the percentages of positive clones (**Table 3**). Taken together, the percentages of positive clones with the ligation of blunt ends, different clone sites, and a Xba I, were approximately 47% ±31% (n=13), 93% ±12% (n=3), and 36% ±10% (n=2) of identified clones, respectively. The efficiencies of blunt-end cloning and Xba I site cloning were not significantly different (47% ±31% vs 36% ±10%, *P>0.05*), In contrast, the efficiencies between blunt-end cloning and different site cloning (47% ±31% vs 93% ±12%), different site cloning and Xba I site cloning (93% ±12% vs 36% ±10%) were significantly different (*P<0.05*). The data of Xba I cloning were consistent with our previous report with BamH I site cloning (**Table 3**)[13].

Here, we reported quantitative models for efficient construction of different vectors, which included different sizes of vectors and inserts, and various clone sites. Firstly, creating clone sites by SDM, or taking advantage of originally existed clone sites of circular vectors, could guarantee that 100% of the linearized DNA fragments were with correct cutting-ends. Secondly, dephosphorylation of vector could confirm that most of the transformants (92.8% for ligations with BamH I site[13], 77% for blunt-end ligations, 93% for ligations with different clone sites, and 100% for ligations with Xba I site, respectively) were with recombinants. Thirdly, optimization of the amount and ratio of the inserts and vectors could increase the rate of monomeric, correct-oriented recombinants (47% ±31%, 93% ±12%, 36% ±10% and 51.3% ±15.2%[13], respectively). Finally, Top10 cells could significantly improve the transformation efficiencies, and therefore facilitate to obtain enough colonies for identification[13]. We designated our optimized method as a "Combinatorial Strategy". With our Strategy, most vectors could be successfully constructed through "one ligation, one transformation, and three to five minipreps". This study provided important quantitative models for efficiently cloning different kinds of vectors with various clone sites, therefore, radically accelerated the biology and medicine research on gene functions both *in vivo* and *in vitro*.

Materials and Methods

Design of SDM primers

SDM was performed to insert Pst I sites for plasmids pcDNA4/β5WT and pcDNA4/β5T1A (Invitrogen, Lab stock) at the 3'-end of β5WT and β5T1A open reading frames, respectively, to create pcDNA4/β5WT/3'-Pst I, and pcDNA4/β5T1A/3'-Pst I vectors. In addition, Xba I sites were inserted by SDM at the 5' and 3' ends of β5T1A open reading frame to create pcDNA4/β5T1A/5'-Xba I/3'-Xba I with pcDNA4/β5T1A as template. All primers, including Pst I and Xba I insertions, were designed according to the guide of Stratagene's QuickChange™ SDM kit, synthesized and purified by Integrated DNA Technologies. For all primers, mutagenized positions were denoted in lower case and underlined.

pcDNA4/3'-Pst I insertion forward:
5'-CATCACCATTGAGTTTAAACctgcagCCGCTGATCAGCCTCGACTG-3';

pcDNA4/3'-Pst I insertion complement:
5'-CAGTCGAGGCTGATCAGCGGctgcagGTTTAAACTCAATGGTGATG-3';

pcDNA4/5'-Xba I insertion forward: 5'-GAATTCTGCAtctagaGATATCCAGC-3';

pcDNA4/5'-Xba I insertion complement: 5'-GCTGGATATCtctagaTGCAGAATTC-3';

pcDNA4/3'-Xba I insertion forward: 5'-CCATTGAGTTTAAACtctagaCCGCTGATCAGCCTC-3';

pcDNA4/3'-Xba I insertion complement: 5'-GAGGCTGATCAGCGGtctagaGTTTAAACTCAATGG-3';

The melting temperatures (T_m, primer-to-template annealing temperature) were calculated by Online Software "Calculate a primer's melting temperature for the QuickChange Site-Directed Mutagenesis Kit" created by David Kim (http://depts.washington.edu/bakerpg/primertemp.html), and primer-pair self-annealing temperatures (T_m^*) were calculated by Integrated DNA Technologies SciTools OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx), respectively (**Table 1**). All the

DNA preparation kits, including Miniprep, Maxiprep, and Gel extraction kits, were purchased from QIAGEN. The DNA purities used in experiments were tested by NanoDrop-1000 Spectrophotometer (NanoDrop Technologies), and all the A260/280 values were ≥ 1.80.

Mutagenesis

The PCR amplifications were carried out with GeneAmp® PCR System 2700 (AB Applied Biosystems). The 50 μl PCR reactions were carried out with 50ng templates, 125ng of each forward and complement primers, 20 μM of each dNTP, 2.5U of PfuUltra DNA polymerase in 1 ×reaction buffer (Statagene). The thermal cycler program for amplifications was as follows: denaturation at 94 ℃ for 2 min; 18 cycles at 94 ℃ for 30 sec, annealing for 30 sec, at 60 ℃ for pcDNA4/β5WT/3'-Pst I and pcDNA4/β5T1A/3'-Pst I insertions, 53 ℃ for pcDNA4/β5WT/5'-Xba I and pcDNA4/β5T1A/5'-Xba I insertions, and 58 ℃ for pcDNA4/β5T1A/5'-Xba I/3'-Xba I insertion, respectively (**Table 2, Figure 2 and 3**), and at 72 ℃ for 7 min; followed by a final extension at 72 ℃ for 10 min. When the amplifications were finished, 1 μl (10U) of Dpn I (Statagene) was added into each reaction, and incubated at 37 ℃ for 1 hour. Finally, 1 μl reaction products were used for transformation into 50 μl DH5α or Top10 competent cells (Invitrogen), respectively, according to the manufacturer's guides. All the transformants were used to spread plates. Transformation colonies were selected and their plasmids were isolated by miniprep, and the positive mutants were identified by sequencing. All the primers are available upon request.

Preparation of vector and insert DNA

LVs, pWPI/Neo (Addgene plasmid 12254, modified by replacing EGFP sequence with Neo, a kind gift from Robert Strome)[13] was digested with Swa I (NEW ENGLAND BioLabs), pLenti CMV/TO Puro DEST (Addgene plasmid 17293)[27] was digested with EcoR V (NEW ENGLAND BioLabs), respectively. and pLVCT-tTR-KRAB (Addgene plasmid 11643)[9] was digested with Pme I and Pst I (NEW ENGLAND BioLabs), sequentially. In addition, pTetO-HGMoPrP (a kind gift from Dr. Prusiner, University of California, San Francisco)[12] was digested with Xba I (NEW ENGLAND BioLabs), and pcDNA4/β5T1A/5'-Xba I/3'-Xba I was digested with Not I and Xho I, simultaneously (NEW ENGLAND BioLabs) (**Figure 1, 2 and 3; Table 3**). All the digested vectors were treated with CIP (NEW ENGLAND BioLabs) at 37 ℃ water bath for 1 hour, respectively, to remove the 5'-phosphate groups[18]. And then, all the vectors were purified by 1% agarose gel electrophoresis to remove CIP, and recovered by QIAGEN Gel Extraction Kit[13].

α-synuclein (Syn) WT and A30P, A53T mutants were cut off from pcNA6 with Pme I, and Rab3A WT, T36N, Q81L mutants, and GDI WT R218E, R240A mutants were cut off from pcDNA3.1 with Pme I (**Figure 1**). The different DNA fragments of β5 WT and T1A mutant were cut off from pcDNA4 with EcoR V and Pme I, EcoR V and Pst I, Not I and Xho I, and Xba I, respectively, for different cloning (**Figure 1, 2 and 3**). Particularly, when β5WT and β5T1A mutant were severed from pcDNA4/3'-Pst I vectors with 5'-EcoR V and 3'-Pst I enzymes, at first, the vectors needed to be digested by EcoR V enzyme, and then with Pst I enzyme, sequentially (**Figure 2, Table 3**). Because, there were a Pst I site at 952 nucleotide and a EcoR V site at 955 nucleotide. As a general rule, when the vectors and inserts were prepared with enzyme digestion, try to avoid using restriction enzymes that cleave within 12bp of each other in the multiple cloning site, or, after one of these sites has been cleaved, the second site will be located too close to the end of the linear DNA to allow efficient cleavage by the second enzyme[18]. All the vectors described above were from lab stocks. Then, all the inserts, α-Syn, Rab3A, GDI, and β5, both WT and mutants were recovered by 1% agarose electrophoresis and gel extraction procedure, respectively.

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Ligation

The ligation reactions were set up according to the molar ratios of inserts and vectors, and DNA amounts, indicated in Table 3. In 10μl ligation systems, the vector and insert were pooled together, warmed at 45℃ for 5 min to melt any cohesive termini that have re-annealed during fragment preparation, then chilled on ice for 2 min, and then 1μl 10×T4 DNA ligase buffer and 0.5μl high-concentration T4 DNA ligase (400U/μl or 2000U/μl, NEW ENGLAND BioLabs) were added[18]. The 10μl reaction mixtures were incubated in GeneAmp® PCR System 2700 at 16℃ for 16 hours followed by inactivation at 65℃ for 10 min, and then set at 4℃ until transformation.

Transformation and identification

2μl (about 30~80ng) volumes of the ligation products were used to transform 50μl Top10 competent cells according to the manufacturer's instructions (**Table 3**). In order to obtain more colonies, all the transformation cells were used to spread plates. Positive colonies were primarily analyzed by restriction endonuclease digestion, and further confirmed by DNA sequencing.

Data statistics

Data were analyzed by Mean ± SD and Student's t-Test.

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Author contributions

G.Z. and A.T. conceived and designed the experiments. G.Z. performed the experiments and carried out data analysis. G.Z. wrote the paper.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Figure legends

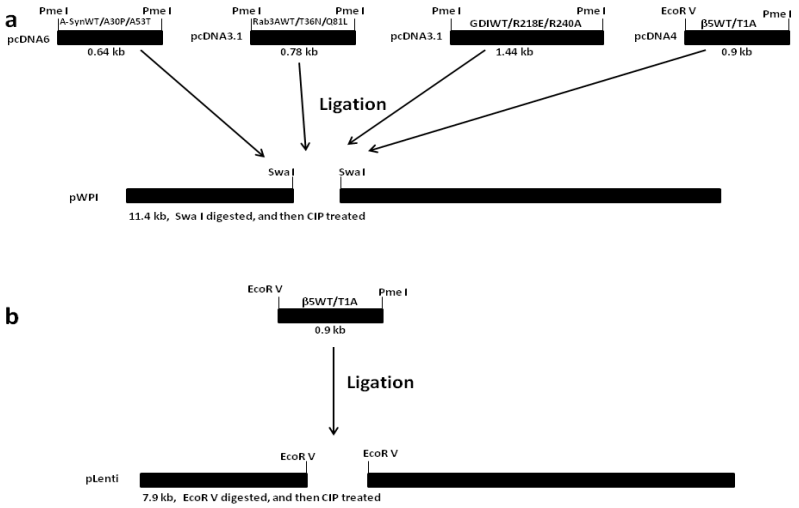

Figure 1 Schematic representation for the cloning of pWPI and pLenti LVs

a. α-Syn WT and A30P, A53T mutants were cleaved from pcDNA6 with Pme I restriction endonuclease. Rab3A WT and T36N, Q81L mutants, and GDI WT and R218E, R240A mutants, were also digested with Pme I enzyme from pcDNA3.1. β5 WT and T1A mutant were cleaved from pcDNA4 with 5'-EcoR V and 3'-Pme I enzymes. The pWPI vector was digested with Swa I enzyme, followed by CIP treatment. EcoR V, Pme I and Swa I were blunt-end cleavage endonuclease enzymes, therefore, the inserts could be ligated into the pWPI vector.

b. Inducible vector pLenti (pLenti CMV/TO Puro DEST) was digested with EcoR V enzyme, and followed by CIP treatment, and then the inserts β5 WT and T1A were ligated with pLenti to clone the inducible LV pLenti/β5WT and pLenti/β5T1A.

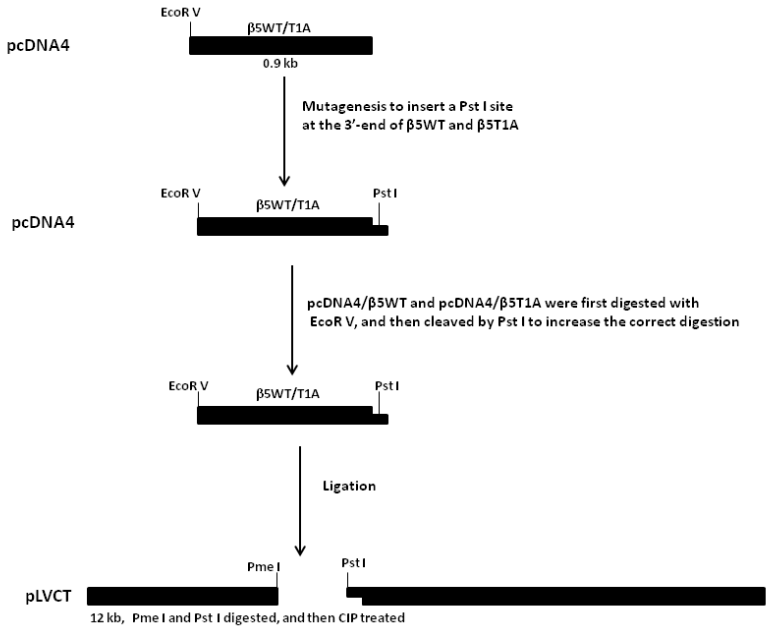

Figure 2 Schematic representation for the cloning of pLVCT inducible LVs

The EGFP fragment could be cut off by a 5'-Pme I and a 3'-Pst I clone sites in the original pLVCT (pLVCT-tTR-KRAB) vector. To clone pLVCT/β5WT and pLVCT/β5T1A inducible vectors, a Pst I clone site was inserted at the 3'-end of β5 WT and T1A mutant taken pcDNA4/β5WT and pcDNA4/β5T1A plasmids as templates by SDM. Then the inserts were, at first, cleaved from pcDNA4 by EcoR V, and then by Pst I, sequentially. The pLVCT vector was treated with CIP following Pme I and Pst I digestion, and the EGFP fragment was replaced by β5 WT and T1A mutant, respectively.

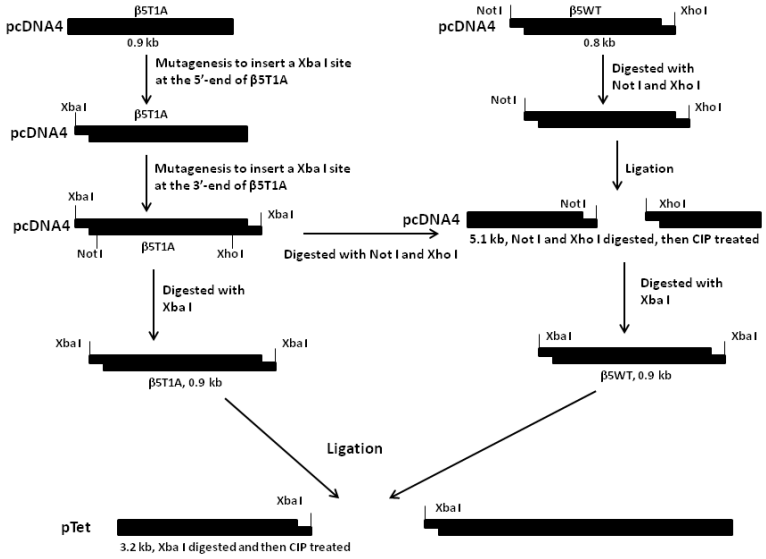

Figure 3 Schematic representation for the cloning of inducible pTet/β5WT and pTet/β5T1A vectors

Two Xba I sites were sequentially inserted at the 5', and 3'-ends of β5T1A, taken pcDNA4/β5T1A plasmid as template, by SDM to form pcDNA4/β5T1A/5'-Xba I/3'-Xba I. pcDNA4/β5WT plasmid was digested by Not I and Xho I, simultaneously, to recover 5'-Not I-β5WT-3'-Xho I fragment. At the same time, pcDNA4/β5T1A/5'-Xba I/3'-Xba I plasmid was digested with Not I and Xho I, followed by the treatment with CIP, to recover dephosphorylated Not I-pcDNA4/5'-Xba I/3'-Xba I-Xho I vector fragment. And then, the insert and vector were ligated together to construct pcDNA4/β5WT/5'-Xba I/3'-Xba I vector. Finally, both of them were digested with Xba I to recover 5'-Xba I-β5WT/T1A-3'-Xba I inserts, and the pTet original vector was also cleaved by Xba I, followed by CIP treatment, to construct pTet/β5WTand pTet/β5T1A inducible expression vectors using Xba I clone site.

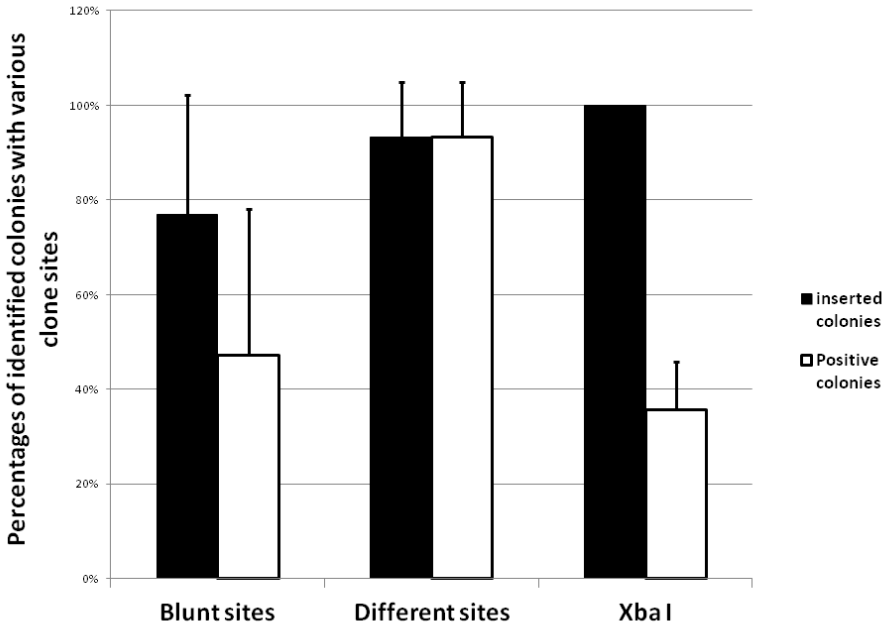

Figure 4 Comparison of the cloning efficiencies with various clone sites

The percentages of inserted colonies and positive colonies between Blunt sites/Different sites, Blunt sites/Xba I site, Different sites/Xba I site were analyzed by Mean ±SD and Student's t-Test. The percentages of inserted clones with ligation of blunt-end sites, different clone sites, and a Xba I site, were approximately 77% ±25% (n=13), 93% ±12% (n=3), and 100% ±0 (n=2) of identified clones, respectively. Among them, the efficiencies between blunt sites and Xba I site were significantly different (*P=0.0064<0.05*). Whereas, the differences between blunt sites/different sites and different sites/Xba I site were not significantly different (*P=0.1304 vs P=0.4226, P>0.05*). On the other hand, the percentages of positive clones with ligation of blunt-end sites, different clone sites, and a Xba I site, were approximately 47% ±31% (n=13), 93% ±12% (n=3), and 36% ±10% (n=2), respectively. Among them, the efficiencies between blunt sites/Xba I site and different sites/Xba I site were not significantly different (*P=0.3468 vs P=0.0154, P>0.05*). In contrast, the efficiencies between blunt sites/different sites cloning were significantly different (*P=0.0018<0.05*).

Tables

Table 1 Characteristics of mutagenesis primer pairs

Primer name	Length/mutation (bases)	T_m (℃)	T_m* (℃)
pcDNA4/3'-Pst I insertion forward	46/6	75.2	69.1
pcDNA4/3'-Pst I insertion complement	46/6	75.2	69.1
pcDNA4/5'-Xba I insertion forward	26/6	49.8	54.9
pcDNA4/5'-Xba I insertion complement	26/6	49.8	54.9
pcDNA4/3'-Xba I insertion forward	36/6	65.4	63.3
pcDNA4/3'-Xba I insertion complement	36/6	65.4	63.3

Note: T_m: primer-to-template annealing temperature, which considered the mismatches of the bases; T_m^*: primer-pair self-annealing temperatures.

Table 2 Mutagenesis efficiency of Pst I and Xba I insertion

Mutagenesis (insertion)	Annealing temperature (℃)	Hosts	Transformed colonies	Sequenced colonies	Positive colonies
pcDNA4/β5WT/3'-Pst I	60	Top10	4 (n=1)	2	1 (50%)
pcDNA4/β5T1A/3'-Pst I	60	DH5α	5 (n=1)	3	1 (33.3%)
pcDNA4/β5WT/5'-Xba I*	53	Top10	~50 (n=3)	18	0
pcDNA4/β5T1A/5'-Xba I	53	Top10	16 (n=1)	10	1 (10%)
pcDNA4/β5T1A/5'-Xba I/3'-Xba I	58	Top10	8 (n=1)	8	4 (50%)

* Note: One PCR mutagenesis reaction was run, and three transformations were performed.

Table 3 Construction efficiencies of different vectors with various clone sites

Vector sizes (kb) & clone sites (5'-, 3'-)	Insert sizes (kb) & clone sites (5'-, 3-')	Inserts/vectors, DNA concentration (ng/μl)	Transformed colonies	Identified colonies	Inserted colonies	Positive colonies	Data analyses (Mean ±SD)
pWPI (11.4, Swa I)	α-Syn-WT (0.64, Pme I)	2 : 1 (39.2)	13 (n=1)	4 (n=1)	3 (75%)	1 (25%)	47% ±31%
pWPI (11.4, Swa I)	α-Syn-A30P (0.64, Pme I)	1.8 : 1 (38.8)	7 (n=1)	4 (n=1)	4 (100%)	3 (75%)	
pWPI (11.4, Swa I)	α-Syn-A53T (0.64, Pme I)	3.4 : 1 (41.9)	10 (n=1)	4 (n=1)	1 (25%)	1 (25%)	
pWPI (11.4, Swa I)	Rab3A-WT (0.78, Pme I)	3.2 : 1 (20.3)	2 (n=1)	2 (n=1)	2 (100%)	2 (100%)	
pWPI (11.4, Swa I)	Rab3A-T36N (0.78, Pme I)	3.5 : 1 (20.7)	14 (n=1)	10 (n=1)	8 (80%)	2 (20%)	
pWPI (11.4, Swa I)	Rab3A-Q81L (0.78, Pme I)	1.9 : 1 (18.9)	11 (n=1)	4 (n=1)	4 (100%)	3 (75%)	
pWPI (11.4, Swa I)	GDI-WT (1.44, Pme I)	1.7 : 1 (26.2)	13 (n=1)	6 (n=1)	4 (66.7%)	3 (50%)	
pWPI (11.4, Swa I)	GDI-R218E (1.44, Pme I)	1.8 : 1 (24.5)	7 (n=1)	5 (n=1)	2 (40%)	1 (20%)	
pWPI (11.4, Swa I)	GDI-R240A (1.44, Pme I)	2.4 : 1 (28.0)	10 (n=1)	4 (n=1)	4 (100%)	1 (25%)	
pWPI (11.4, Swa I)	β5-WT (0.9, EcoR V, Pme I)	2.1 : 1 (23.3)	20 (n=1)	2 (n=1)	2 (100%)	2 (100%)	
pWPI (11.4, Swa I)	β5-T1A (0.9, EcoR V, Pme I)	1.7 : 1 (19.5)	2 (n=1)	2 (n=1)	1 (50%)	1 (50%)	
pLenti (7.9, EcoR V)	β5-WT (0.9, EcoR V, Pme I)	22 : 1 (18.9)	12 (n=1)	8 (n=1)	6 (75%)	3 (37.5%)	
pLenti (7.9, EcoR V)	β5-T1A (0.9, EcoR V, Pme I)	14.1 : 1 (14.0)	13 (n=1)	8 (n=1)	7 (87.5%)	1 (12.5%)	
pLVCT (12, Pme I, Pst I)	β5-WT (0.9, EcoR V, Pst I)	3 : 1 (17.6)	~300 (n=1)	5 (n=1)	4 (80%)	4 (80%)	93% ±12%
pLVCT (12, Pme I, Pst I)	β5-T1A (0.9, EcoR V, Pst I)	3 : 1 (17.6)	~100 (n=1)	5 (n=1)	5 (100%)	5 (100%)	
pcDNA4 (5.1, Not I, Xho I)	β5-WT (0.8, Not I, Xho I)	1.9 : 1 (28.1)	~500 (n=1)	8 (n=1)	8 (100%)	8 (100%)	
pTet (3.2, Xba I)	β5-WT (0.9, Xba I)	2.1 : 1 (21.9)	~1000 (n=1)	14 (n=1)	14 (100%)	4 (28.6%)	36% ±10%
pTet (3.2, Xba I)	β5-T1A (0.9, Xba I)	1.5 : 1 (19.7)	~1000 (n=1)	14 (n=1)	14 (100%)	6 (42.9%)	

Notes: Percentages of positive colonies between:

Blunt sites/different sites: P=0.0018; Blunt sites/Xba I: P=0.3468; Different sites/Xba I: P=0.0154.

Percentages of inserted colonies between:

Blunt sites/Different sites: P=0.1304; Blunt sites/Xba I: P=0.0064; Different sites/Xba I: P=0.4226.