

“Combinatorial Strategy”: A Highly Efficient Method for Cloning Different Vectors with Various Clone Sites

Gang Zhang^{1,2,*}, Anurag Tandon¹

¹Department of Medicine, Centre for Research in Neurodegenerative Diseases, University of Toronto, Toronto, Canada

²Division of Nephrology, Massachusetts General Hospital, Charlestown, USA

*Corresponding author: gang.zhang@utoronto.ca; sdzbzhanggang@yahoo.com

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Abstract In this study, we generalized the “Combinatorial Strategy” for efficient cloning of different vectors with various clone sites. 1) Using originally existed clone sites from circular plasmids to prepare the inserts, if no appropriate sites available, performing SDM to create compatible sites, could achieve maximal correct digestion of the inserts. 2) Different vectors were digested with various restriction endonucleases, and then dephosphorylated after digestion. 3) Top10 competent cells were used for transformation to increase the transformant colonies. Our results showed that, when either blunt-sites or 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, were used, the percentages of positive clones were nearly 100%. With this strategy, most vectors could be successfully cloned through “one ligation, one transformation, three to five minipreps”.

Keywords: DNA recombination, combinatorial strategy, vector cloning, site-directed mutagenesis, Top 10 cells, clone sites

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1. 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 biomedical research 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 gradually. Therefore, there is an increasing need for efficient strategies to clone genes of interest into various expression vectors, precisely and efficiently, according to different aims. For example, to establish stable transgene cell lines, lentiviral vectors (LVs) might be good choices. And to achieve conditional transgene over-expression, drug-inducible systems were designed for this purpose [3,4,5,6,7,8,9,10,11,12].

Generally, the DNA recombination techniques were of low efficiency. To improve the efficiencies of plasmid vector cloning, recently, we reported an efficient method, “Combinatorial strategy”, for lentiviral vector cloning 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 [14,15,16]. Currently, the QuikChange™ SDM System developed by Stratagene is a commonly adopted kit for mutagenesis using double-

stranded plasmid DNAs as templates. The advantage of this strategy is that the products after mutagenesis are circular, double-stranded plasmid DNAs. 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 laboratory focused on Parkinson disease, we need to clone several different kinds of expression plasmid vectors for α -synuclein (α -Syn) and related genes, such as Rab3A, GDI, β 5, 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 or EcoR V, and Pst I), two different overhang-end sites (Not I and Xho I), and one overhang-end site (Xba I), and so on. Encouraged by our former report [13], with our efficient 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 $47\% \pm 31\%$ (n=13), $93\% \pm 12\%$ (n=3), and $36\% \pm 10\%$ (n=2), respectively. This study provided important quantitative models for efficient construction of different plasmid vectors, and laid significant foundations for gene functional analyses.

2. Materials and Methods

2.1. 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 site was inserted by SDM at the 5'- and 3'- ends of β5T1A open reading frame, respectively, 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'-CATCACCATTGAGTTTAAACctgcagCCGCTGATCA GCCTCGACTG-3';

pcDNA4/3'-Pst I insertion complement: 5'-CAGTCGAGGCTGATCAGCGGctgcagGTTTAAACTC AATGGTGATG-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'-GAGGCTGATCAGCGtctagaGTTTAAACTCAATGG-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 .

Table 1. Characteristics of mutagenesis primer pairs

Primer name	Length/mutation (bases)	T_m (°C)	T_m^* (°C)
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.

2.2. Mutagenesis

The PCR amplifications were carried out with GeneAmp® PCR System 2700 (AB Applied Biosystems). The 50µl PCR reactions were carried out with 50ng templates, 125ng of each forward and complement primers, 20µM of each dNTP, 2.5U of PfuUltra DNA polymerase in 1×reaction buffer (Stratagene). The thermal cycler program for amplifications was as follows: denaturation at 94°C for 2 min; 18 cycles at 94°C for 30 sec, annealing for 30 sec, at 60°C for pcDNA4/β5WT/3'-Pst I and pcDNA4/β5T1A/3'-Pst I insertions, 53°C for pcDNA4/β5WT/5'-Xba I and pcDNA4/β5T1A/5'-Xba I insertions, and 58°C for pcDNA4/β5T1A/5'-Xba I/3'-Xba

I insertion, respectively (Table 2, Figure 2 and Figure 3), and at 72°C for 7 min; followed by a final extension at 72°C for 10 min. When the amplifications were finished, 1µl (10U) of Dpn I (Stratagene) was added into each reaction, and incubated at 37°C for 1 hour. Finally, 1µl reaction products were used for transformation into 50µl 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.

Table 2. Mutagenesis efficiency of Pst I and Xba I insertion

Mutagenesis (insertion)	Annealing temperature (°C)	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.

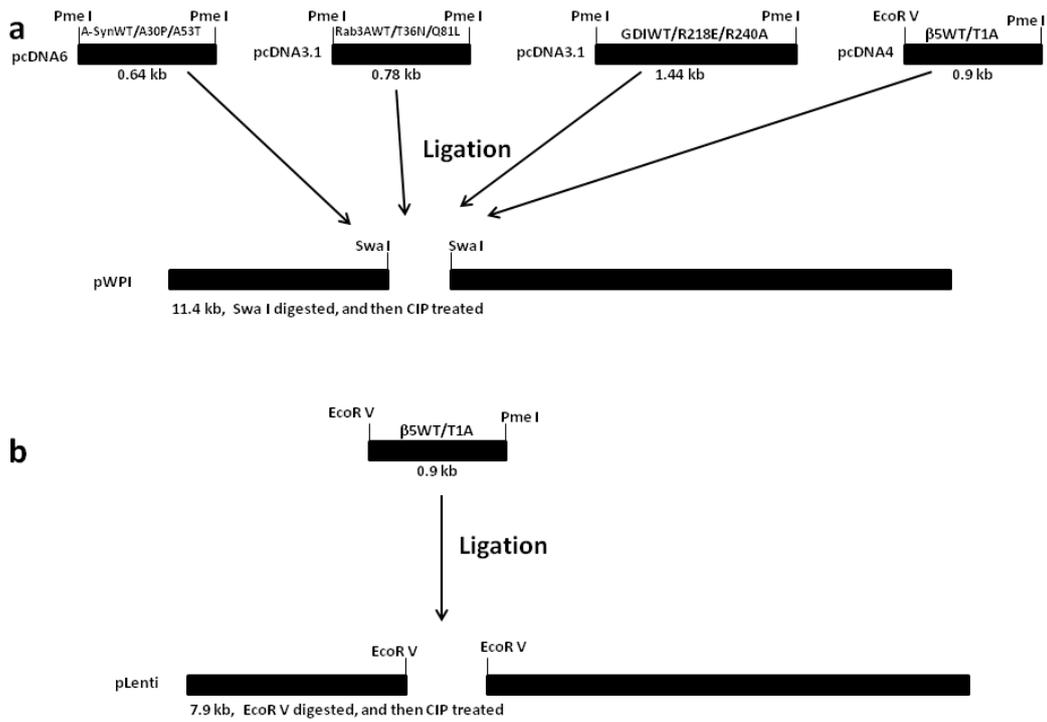


Figure 1. Schematic representation for the cloning of pWPI and pLenti LVs

- 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.

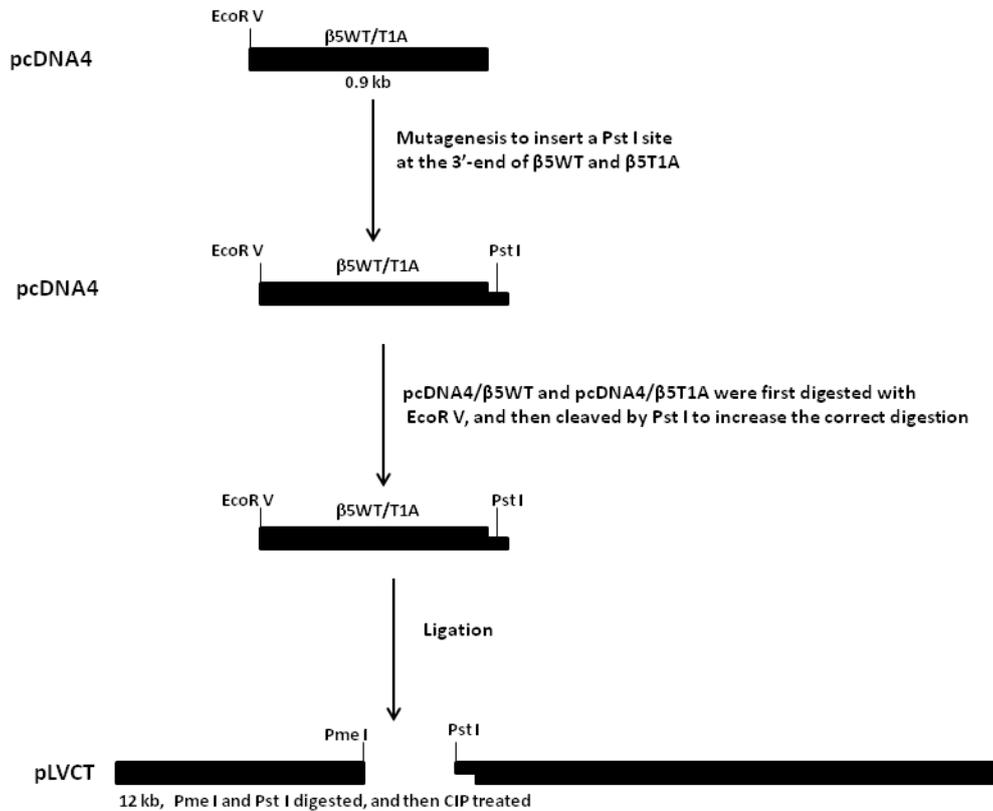


Figure 2. Schematic representation for the cloning of pLVCT inducible LVs

The EGFP fragment could be cut off by 5'-Pme I and 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, respectively, 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.

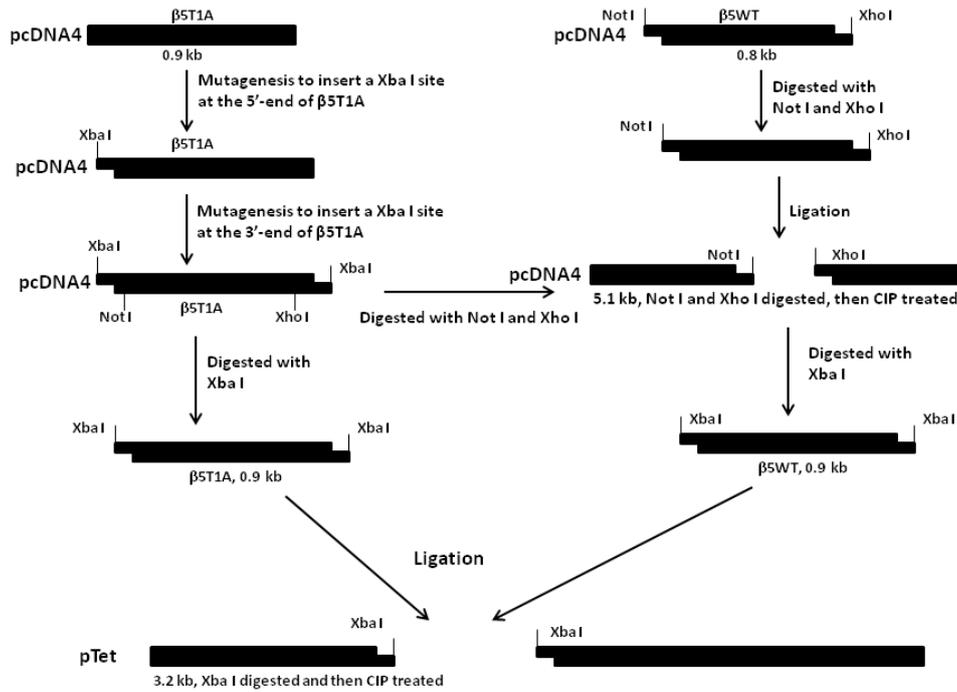


Figure 3. Schematic representation for the cloning of inducible pTet/β5WT and pTet/β5T1A vectors

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/β5WT and pTet/β5T1A inducible expression vectors using Xba I clone site.

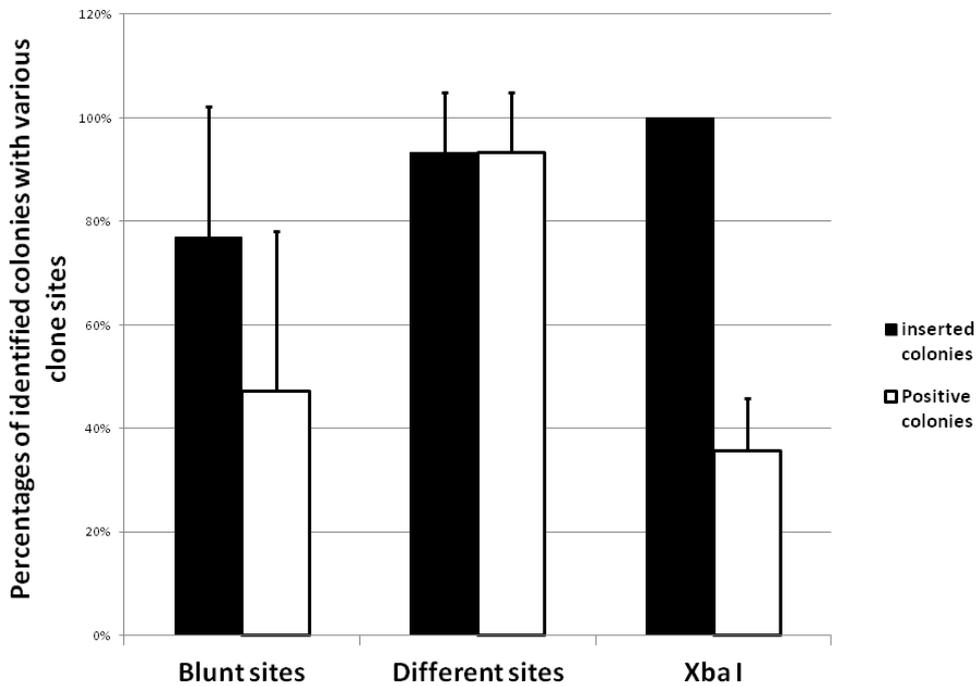


Figure 4. Comparison of the cloning efficiencies with various clone sites

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), 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 ($P=0.1304$, and $P=0.4226$, $P > 0.05$). 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 ($P=0.3468$, and $P=0.0154$, $P > 0.05$). In contrast, the efficiencies between blunt sites/different sites cloning were significantly different ($P=0.0018 < 0.05$).

2.3. Preparation of Vector and Insert DNA

LVs, pWPI/Neo (Addgene plasmid 12254, modified by replacing EGFP sequence with Neo) [13] was digested with Swa I (NEW ENGLAND BioLabs), pLenti CMV/TO Puro DEST (Addgene plasmid 17293) [18] 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-HGMPPrP (a kind gift from Dr. Prusiner, University of California, San

Francisco) [12] was digested with Xba I (NEW ENGLAND BioLabs), and pcDNA4/ β 5T1A/5'-Xba I/3'-Xba I was digested with Not I and Xho I, simultaneously (NEW ENGLAND BioLabs) (Figure 1, Figure 2 and Figure 3; Table 3). All the digested vectors were treated with CIP (NEW ENGLAND BioLabs) at 37°C water bath for 1 hour, respectively, to remove the 5'-phosphate groups [19]. And then, all the vectors were purified by 1% agarose gel electrophoresis to remove CIP, and recovered by QIAGEN Gel Extraction Kit [13].

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/ μ l)	Transformed colonies	Identified colonies	Inserted colonies	Positive colonies	Data analyses (Mean \pm 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% \pm 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)	β 5-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)	β 5-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)	β 5-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)	β 5-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)	β 5-WT (0.9, EcoR V, Pst I)	3 : 1 (17.6)	~300 (n=1)	5 (n=1)	4 (80%)	4 (80%)	93% \pm 12%
pLVCT (12, Pme I, Pst I)	β 5-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)	β 5-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)	β 5-WT (0.9, Xba I)	2.1 : 1 (21.9)	~1000 (n=1)	14 (n=1)	14 (100%)	4 (28.6%)	36% \pm 10%
pTet (3.2, Xba I)	β 5-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: $P=0.4226$.

α -synuclein (Syn) WT and A30P, A53T mutants were cut off from pcDNA6 with Pme I. Rab3A WT, and T36N, Q81L mutants, GDI WT and R218E, R240A mutants were cut off from pcDNA3.1 with Pme I as well (Figure 1). The different DNA fragments of β 5 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, Figure 2 and Figure 3). Particularly, when β 5WT and β 5T1A mutant were severed from pcDNA4/3'-Pst I vectors with 5'-EcoR V and 3'-Pst I enzymes, at first, the vectors were 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 [19]. All the vectors described above, except those particularly noted, were from lab stocks. Finally, all the inserts, α -Syn, Rab3A, GDI, and β 5, both WT and mutants, were recovered by 1% agarose electrophoresis and gel extraction procedure, respectively.

2.4. Ligation

The ligation reactions were set up according to the molar ratios of inserts and vectors, and DNA amounts, indicated in Table 3. In 10 μ l ligation systems, the vector and insert were pooled together, warmed at 45°C for 5 min to melt any cohesive termini that have re-annealed during fragment preparation, then chilled on ice for 2 min, and then 1 μ l 10 \times T4 DNA ligase buffer and 0.5 μ l high-

concentration T4 DNA ligase (400U/ μ l or 2000U/ μ l, NEW ENGLAND BioLabs) were added [19]. The 10 μ l reaction mixtures were incubated in GeneAmp® PCR System 2700 at 16°C for 16 hours followed by inactivation at 65°C for 10 min, and then set at 4°C until transformation.

2.5. Transformation and Identification

2 μ l (about 30~80ng) volumes of the ligation products were used to transform 50 μ l 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.

2.6. Data statistics

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

3. Results

3.1. Pst I and Xba I insertion by SDM

SDM was performed by PCR, and the parent template DNAs were digested with Dpn I. Different annealing temperatures were used for pcDNA4/ β 5WT/3'-Pst I, pcDNA4/ β 5T1A/3'-Pst I, pcDNA4/ β 5TW/5'-Xba I, pcDNA4/ β 5T1A/5'-Xba I, and pcDNA4/ β 5T1A/5'-Xba I/3'-Xba I insertions, representing approximately $T_m^* - 9^\circ\text{C}$, $T_m + 3^\circ\text{C}$, and $T_m^* - 5^\circ\text{C}$ (Table 1 and Table 2; Figure 2 and Figure 3), respectively. After transformation into Top10 or DH5 α cells and identified by sequencing, our results showed that, the mutagenesis efficiencies were approximately 50% for pcDNA4/ β 5WT/3'-Pst I insertion,

33.3% for pcDNA4/ β 5T1A/3'-Pst I insertion, 10% for pcDNA4/ β 5T1A/5'-Xba I insertion, and 50% for pcDNA4/ β 5T1A/5'-Xba I/3'-Xba I insertion, respectively. In addition, positive mutagenesis clones were not obtained for pcDNA4/ β 5WT/5'-Xba I insertion after sequencing 18 minipreps (Table 1 and Table 2). These data demonstrated that the majority of our designed mutagenesis was successfully achieved.

3.2. Efficient Cloning of Different Vectors with Various Clone Sites

3.2.1. 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 [19,20,21]. Insert (α -Syn WT and A30P, A53T mutants; Rab3A WT and T36N, Q81L mutants; GDI WT and R218E, R240A mutants; β 5 WT and T1A mutant; respectively) and pWPI/Neo vector DNAs were pooled together in 10 μ l ligation reactions with DNA concentrations around 18.9~42ng/ μ l, and insert to vector molar ratios about 1~3.5:1 (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 endonuclease digestion and sequencing, the percentages of positive clones with monomeric, correct-oriented inserts were ranging from 20% to 100%, respectively (Table 3). Except the cloning of pWPI/GDIWT/Neo, all the blunt-end cloning described above was achieved by performing one ligation and one transformation. For 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 10 μ l ligation system, the molar ratios of insert β 5 WT and T1A mutant to pLenti vector were 22:1 and 14.1:1, and the DNA amounts were about 18.9ng/ μ l and 14ng/ μ l, 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% \pm 25%, and 47% \pm 31%, respectively (Table 3, Figure 4).

3.2.2. 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 10 μ l ligation system, the molar ratios of inserts β 5WT and T1A mutant to pLVCT vector were around 3:1, and the DNA

amounts were around 17.6ng/ μ l, respectively (Table 3, Figure 2). After transformation into Top10 cells, about 300 and 100 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/ β 5WT (Table 2), pcDNA4/ β 5T1A/5'-Xba I/3'-Xba I was digested with Not I and Xho I, simultaneously, and followed by CIP treatment, the insert β 5WT (digested with Not I and Xho I) was ligated into the vector pcDNA4/5'-Xba I/3'-Xba I to form pcDNA4/ β 5WT/5'-Xba I/3'-Xba I (Figure 3). The molar ratio of insert β 5WT to pcDNA4/5'-Xba I/3'-Xba I vector was about 1.9:1, and the DNA amount was around 28.1ng/ μ l, respectively. After transformation into Top10 cells, about 500 colonies were obtained, the percentages of inserted clones and positive clones were both 100% (Table 3).

3.2.3. Construction of Inducible pTet/ β 5WT and pTet/ β 5T1A Vectors with a Unique 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. β 5WT and β 5T1A inserts were cleaved from vectors pcDNA4/5'-Xba I/3'-Xba I, and ligated with pTet, to form inducible vector pTet/ β 5WT and pTet/ β 5T1A (Figure 3). In 10 μ l ligation system, the molar ratios of insert β 5WT and T1A mutant to pTet vector were about 2.1:1 and 1.5:1, and the DNA amounts were about 21.9ng/ μ l and 19.7ng/ μ l, 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, among the identified clones, 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 or Pme I and Pst I; Not I and Xho I), and a Xba I site (Figure 1, Figure 2 and Figure 3), were approximately 77% \pm 25% and 47% \pm 31% (n=13), 93% \pm 12% and 93% \pm 12% (n=3), 100% \pm 0 and 36% \pm 10% (n=2), respectively. Among them, the efficiencies of blunt-end cloning and Xba I site cloning were not significantly different (47% \pm 31% vs 36% \pm 10%, $P>0.05$), these data were similar with our previous report with a BamH I clone site¹³ (Table 3). In contrast, the efficiencies between blunt-end cloning and different site cloning (47% \pm 31% vs 93% \pm 12%), different site cloning and Xba I site cloning (93% \pm 12% vs 36% \pm 10%) were significantly different ($P<0.05$).

4. Discussion

In our former report, SDM was performed to insert BamH I clone site for the inserts, hPlk2 WT and mutants and EGFP gene, to efficiently construct LVs [13]. In this study, Pst I clone site at the 3'-ends of β 5 WT and T1A mutant genes was inserted by SDM, to achieve the construction of inducible LVs, pLVCT/ β 5WT and pLVCT/ β 5T1A, with 5'-blunt end and 3'-Pst I as clone

sites. In addition, Xba I site was also inserted at the 5'- and 3'- ends of β 5T1A mutant, respectively, to construct the inducible expression vector pTet/ β 5WT and pTet/ β 5T1A (Table 1 and Table 2). The Stratagene SDM protocol suggested that the prime pairs should be of 25~45 bases in length with melting temperatures (T_m) $\geq 78^\circ\text{C}$. Compared with our previous report [13], in this study, prime pairs with shorter length (ranging from 26 to 46 bases) and lower T_m (ranging from 49.8 to 75.2 $^\circ\text{C}$), were adopted for SDM (Table 1). And the efficiencies of mutagenesis were lower (from 10% to 50%) than our former report (Table 2). These data suggested that longer prime pairs with higher T_m might be more efficient for clone site insertion than shorter ones [13] (Table 1 and Table 2). Although SDM is a powerful tool to change DNA sequences at specific positions in genetic engineering and clone site insertion, under some circumstances, the QuickChangeTM SDM system of Stratagene could lead to lower PCR efficiency, and even the failure of mutagenesis. In these cases, some modifications would be made to circumvent the problems, such as using primers containing extended non-overlapping sequences at the 3'-end and primer-primer complementary sequences at the 5'-end, running two PCR reactions in parallel with each one of the forward and reverse primers, and so on [13,15,16,23]. After the insertion of Pst I clone site at the 3'-ends of β 5WT and β 5T1A mutant with SDM, 100% of the linearized 5'-EcoR V- β 5WT/T1A-3'-Pst I DNA fragments were theoretically with correct cutting ends. Whereas, due to the insufficient stable-association of the restriction endonucleases with the extra 3-4 bases at the terminal restriction sites, correct cutting ends could not be confirmed with the method of incorporating of restriction sites into PCR primers [24,25]. 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/ β 5WT, and 100% for pLVCT/ β 5T1A, respectively (Table 3, Figure 2). After Xba I site was inserted at the 5'- and 3'- ends of β 5T1A mutant, respectively, the Tet-inducible expression vectors, pTet/ β 5WT and pTet/ β 5T1A, were successfully constructed with a unique Xba I clone site. The percentages of positive clones were 28.6% and 42.9% ($36\% \pm 10\%$, $n=2$), respectively (Table 3, Figure 3). These data were in agreement with our former report [13].

Ligation is the critical step in plasmid vector cloning, and the number of transformants is radically affected by the ligation efficiencies. 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 [19]. 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 [26,27]. Our data demonstrated this phenomenon. 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. Furthermore, 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).

Finally, 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 β 5 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 $47\% \pm 31\%$ ($n=13$), $93\% \pm 12\%$ ($n=3$), and $36\% \pm 10\%$ ($n=2$) of identified clones, respectively. The efficiencies of blunt-end cloning and Xba I site cloning were not significantly different ($47\% \pm 31\%$ vs $36\% \pm 10\%$, $P>0.05$). In contrast, the efficiencies between blunt-end cloning and different site cloning ($47\% \pm 31\%$ vs $93\% \pm 12\%$), different site cloning and Xba I site cloning ($93\% \pm 12\%$ vs $36\% \pm 10\%$) were significantly different ($P<0.05$). The data of the cloning with a Xba I site were consistent with those of the cloning with a BamH I site (Table 3) [13].

In our previous study, we reported a combinatorial method for efficient cloning of LVs, specifically taking pWPI/Neo as vector backbone, hPlk2 WT, mutants, and EGFP gene as inserts, and BamH I as clone site [13]. In order to generalize our efficient strategy, in this study, we reported quantitative models for efficient construction of different plasmid vectors, which adopted different vectors and inserts, and various clone sites. The sizes of the vector backbones were from about 3.2 to 12kb, and those of the inserts were from about 0.64 to 1.44kb (Table 3). Taken together with our former reported hPlk2 gene (about 2.1kb) [13], the size range of the inserts included most of the protein coding open reading frames. Our data demonstrated that the combinatorial method was applicable for almost all the plasmid vector cloning with various clone sites. We designated our efficient method as "Combinatorial Strategy". With this Strategy, most vectors could be successfully constructed through "one ligation, one transformation, and three to five minipreps". This study provided important quantitative models for efficient cloning of different vectors with various clone sites, and could radically facilitate the functional analyses of genes of interest *in vivo* and *in vitro*.

Competing Interests

The authors declare they have no competing interests.

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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.

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