On Lentiviral Vector Cloning, Titration, and Expression in Mammalian Cells

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This talk based on the following publications:

1. **Gang Zhang*** & Anurag Tandon. Quantitative assessment on the cloning efficiencies of lentiviral transfer vectors with a unique clone site. ***Scientific Reports*****,** 2012, 2: 415

2. **Gang Zhang*** & Anurag Tandon. Quantitative models for efficient cloning of different vectors with various clone sites. ***American Journal of Biomedical Research***, 2013, 1(4): 112-119

3. **Gang Zhang***. A new overview on the old topic: the theoretical analysis of "Combinatorial Strategy" for DNA recombination. ***American Journal of Biomedical Research,*** 3013, 1(4):108-111

4. **Gang Zhang*,** Anurag Tandon. Efficient lentiviral transduction of different mammalian cells. **In preparation.**

Main topics

1. Theoretical design of combinatorial strategy

2. Special examples with BamH I clone site

3. General examples with various clone sites

4. The titration of lentiviral vectors and expression in mammalian cells

Part I: Theoretical design of combinatorial strategy

To explore the quantitative law of recombinant DNA

The birth of recombinant DNA technology

In 1972, Jackson et al. reported the first recombinant DNA, **SV40-*λdvgal*** DNA was created. This work won **Nobel Prize in Chemistry** in 1980 (Jackson, et al. PNAS, 1972, 69: 2904-9).

In 1973, **Cohen, et al.** found, for the first time, that the recombinant DNA could be transformed into E. Coli and biologically functional in the host. Stanford University applied for the first **US patent** on **recombinant DNA** in 1974. This patent was awarded in 1980 (Cohen, et al. PNAS, 1973, 70: 3240-4).

This technology revolutionarily changed the bio-medical research during the past decades.

Achievements in DNA recombination:

Many different vector systems available:

1. Regular vectors: pET, pcDNA, etc.
2. Viral vectors: Adenoviral vectors, retroviral vectors, lentiviral vectors, etc.
3. Bacterium expression vectors, insect expression vectors, mammalian expression vectors, etc.
4. Continual expression vectors, inducible expression vectors, etc.
5. Ubiquitous expression vectors, tissue-specific expression vectors, and so on.

At genome era, more and more gene sequences available

Therefore, in theory, we could very easily put any genes of interest into any vectors, and transfer them into any organisms and tissues, to investigate their functions according to our purposes.

Puzzles in molecular cloning:

Some times, if we are lucky, we could clone a vector easily with 5 to 10 minipreps in 3 days, in other times, if we are not lucky, we might need to make hundreds of minipreps, and waste months for a vector, why?

Possible reasons:

1. **The sizes of the vectors and inserts;**
2. **The preparation methods of the inserts;**
3. **The ligation efficiencies of the clone sites;**
4. **The transformation efficiencies of the host cells, etc.**

Now I want to ask "Could we find a way to clone vectors efficiently, and quantitatively?" The answer is YES!!!

Typical reaction system of ligation

ddH2O	Insert	Vector	10 X ligase buffer	T4 DNA ligase
Add up to 20µl	**~100ng**	**~200ng**	**2µl**	**1µl**

A Mole= **~6.02 X 10^{23}** molecules; Average Molar Weight of A, G, C, T= **~660 g**

1 g=1 X 10^{9} **ng**; 1 mole=1 X 10^{12} **pmoles**

Suppose the insert: **1.5kb**, the vector: **5kb, then**

100ng insert=100ng**/**(660 X 1500 X 2 X 1,000,000,000)ng=0.05pmole X 6.02 X 10^{23}**/**10^{12} =**3.01 X 10^{10}** insert molecules

200ng vector=200ng**/**(660 X 5,000 X 2 X 1,000,000,000)ng=0.03pmole X 6.02 X 10^{23}**/**10^{12}**=1.8 X 10^{10}** vector molecules

So what will happen in this tiny 20µl ligation tube?

Main procedure of recombinant DNA

1. **Choose or create compatible clone sites between the vectors and inserts**
   High efficient clone sites, such as EcoR I, BamH I, EcoR V etc.

2. **Digest and purify the vectors and inserts**
   The purities A260/280≥1.80

3. **Ligation,** high concentration T4 DNA ligase

4. **Transformation,** high efficient competent cells, such as DH5α, Top10

5. **Identification by digestion and sequencing**

Approaches to create compatible clone sites

1. **Design PCR primers contained proper clone sites for the inserts**
2. **Make blunt ends by Klenow fragment and T4 DNA polymerase**
3. **Insert clone sites by site-directed mutagenesis**

Design PCR primers contained proper clone sites for the inserts

Advantages: easy and simple, suitable for small size regular cloning

Disadvantages: not guarantee 100% correct-cutting ends, not suitable for large size cloning

From online

Making blunt ends for the inserts or/and vectors with Klenow fragment or T4 DNA polymerase

Functions of Klenow fragment and T4 DNA polymerase:

1. **Fill-in of 5'-overhangs to form blunt ends**
2. **Removal of 3'-overhangs to form blunt ends**
3. **Result in recessed ends due to the 3' to 5' exonuclease activity of the enzymes.**

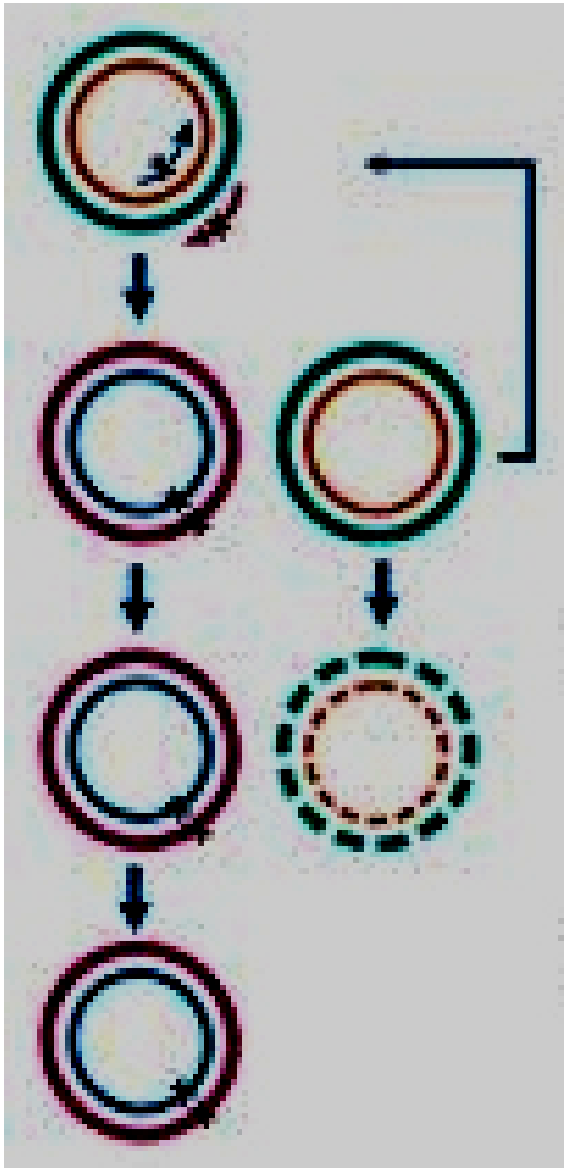
Advantages:

Easy and simple, only a short time reaction, such as 5 to 15 minutes, suitable for easy cloning

Disadvantages:

Not guarantee 100% with the correct blunt ends, not suitable for low efficient cloning

Inserting clone sites by site-directed mutagenesis (SDM)



1. Mutant strand synthesis

Perform thermal cycling to

A. Denature DNA template

B. anneal mutagenic primers containing desired mutantion

C. extend and incorporate primers with PfuUltra DNA polymerase

2. Dpn I digestion of template

Digest parental methylated and hemimethylated DNA with Dpn I

3. Transformation

Transform mutated molecules into competent cells for nick repair

Stratagene SDM Kit

Advantages of inserting clone sites by SDM

1. The mutated products are circular double-stranded plasmid DNA

2. The linearized inserts are theoretically 100% with correct-cutting ends

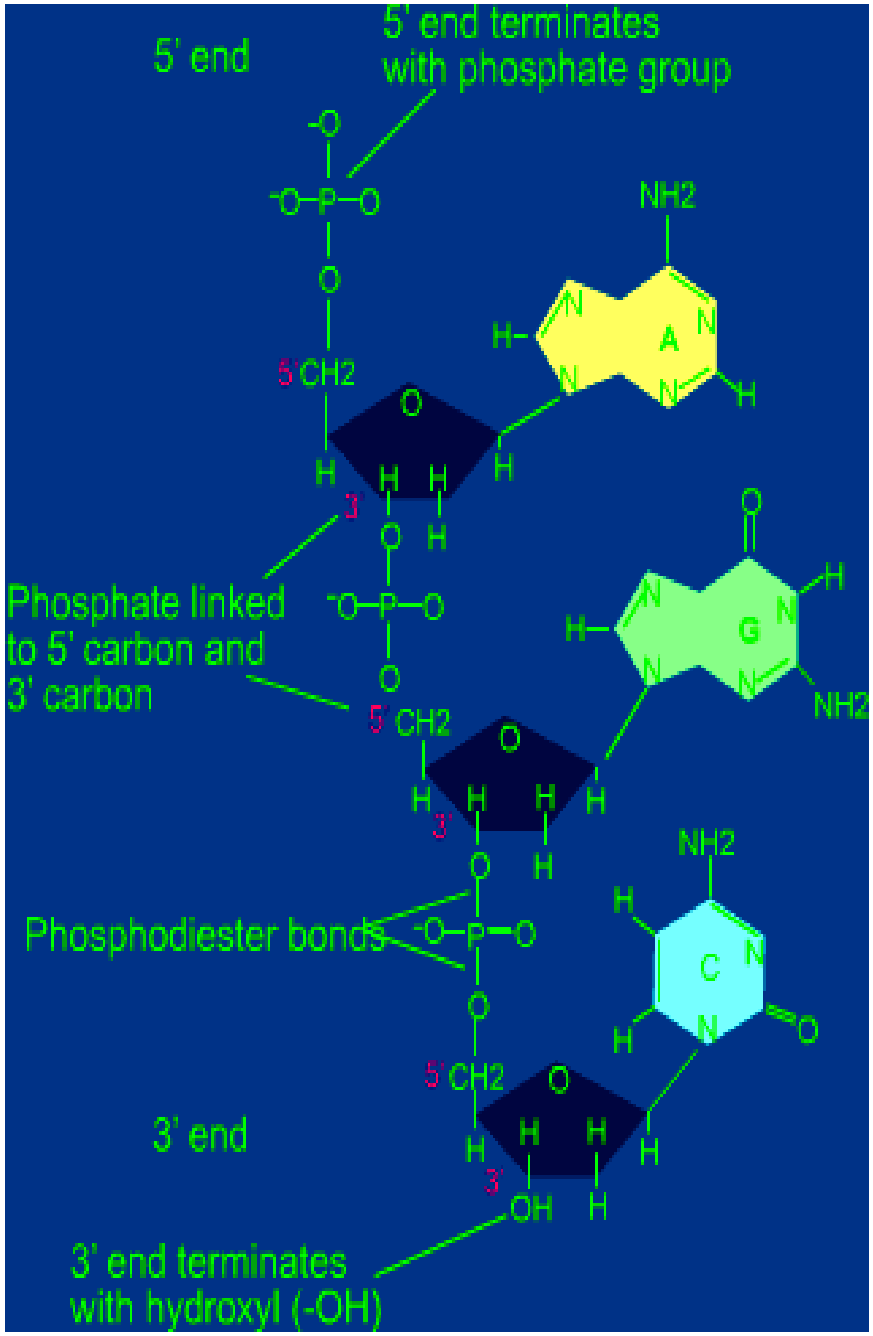
3. Maximal ligation could achieve with the vectors

4. Suitable for low efficient vector cloning, such as lentiviral vectors.

The function of T4 DNA ligase

1. To catalyze the formation of **3’, 5’-Phosphodiester Bond** between juxtaposed 5’**-phosphate groups** and 3’**-hydroxyl groups**.

2. Ligation could take place when there are **mismatches** at or close to the ligation junctions. That is to say, T4 DNA ligase could catalyze the ligation between **different clone sites** (Haarada & Orgel, *Nucl. Acids Res.*, 1993, 21: 2287-91).

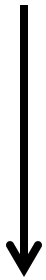


Procedure of regular ligation

1. Inter-molecular reaction to form **non-covalently bonded, linear vector-insert** Hybrids.

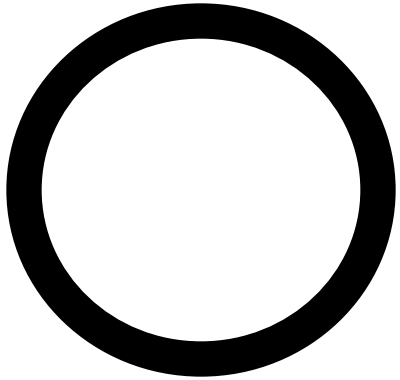
This reaction requires **high DNA concentrations**

2. Intra-molecular reaction to form **non-covalently bonded, circular** molecules.



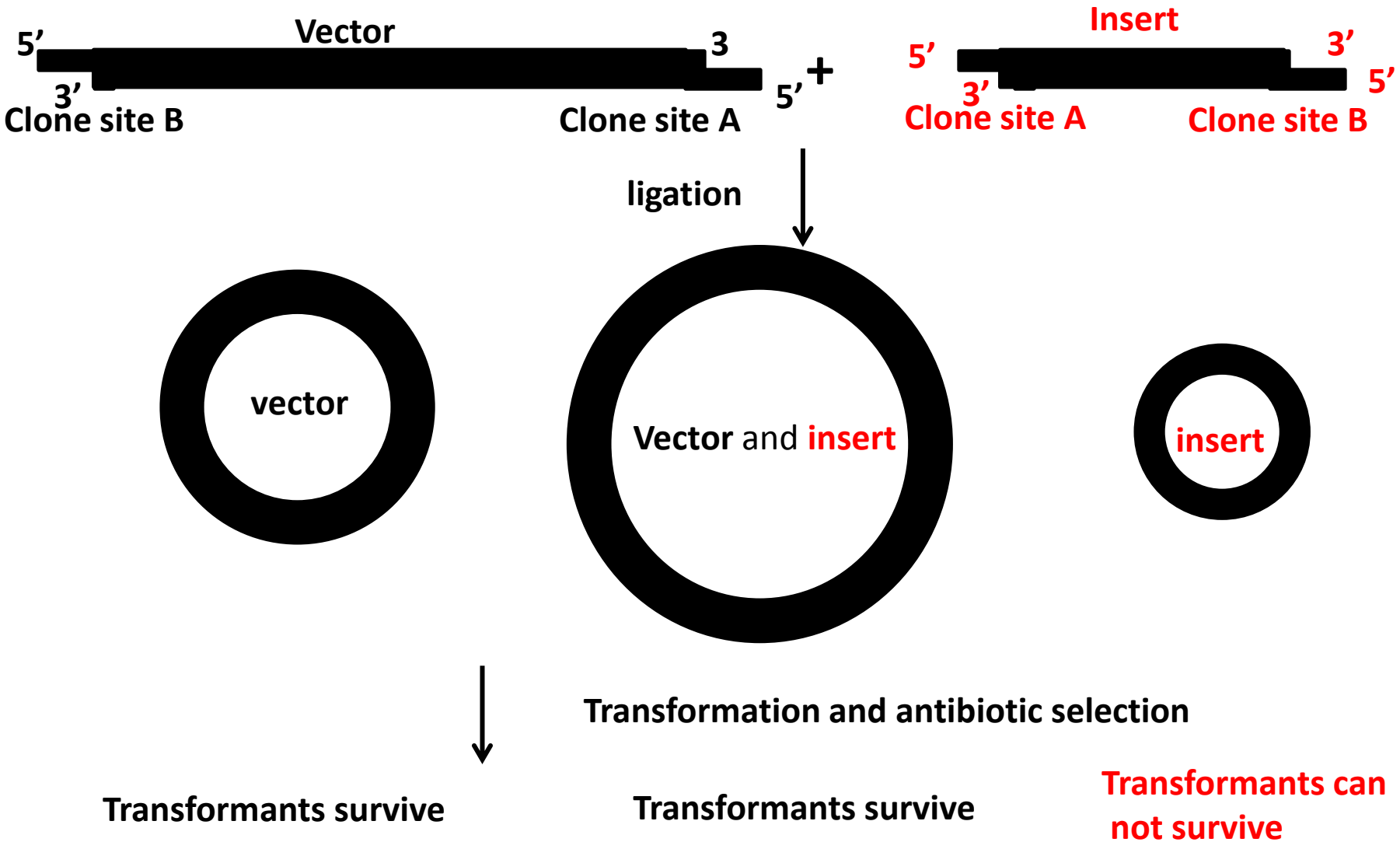
This reaction works efficiently with **low DNA concentrations.**

3. Annealing between the inter and intra molecules brings the 5'-phosphate and 3'-hydroxyl residues of the vectors and inserts into **close alignment**, which allows T4 DNA ligase to catalyze the formation of **3', 5'-phosphodiester bonds.**



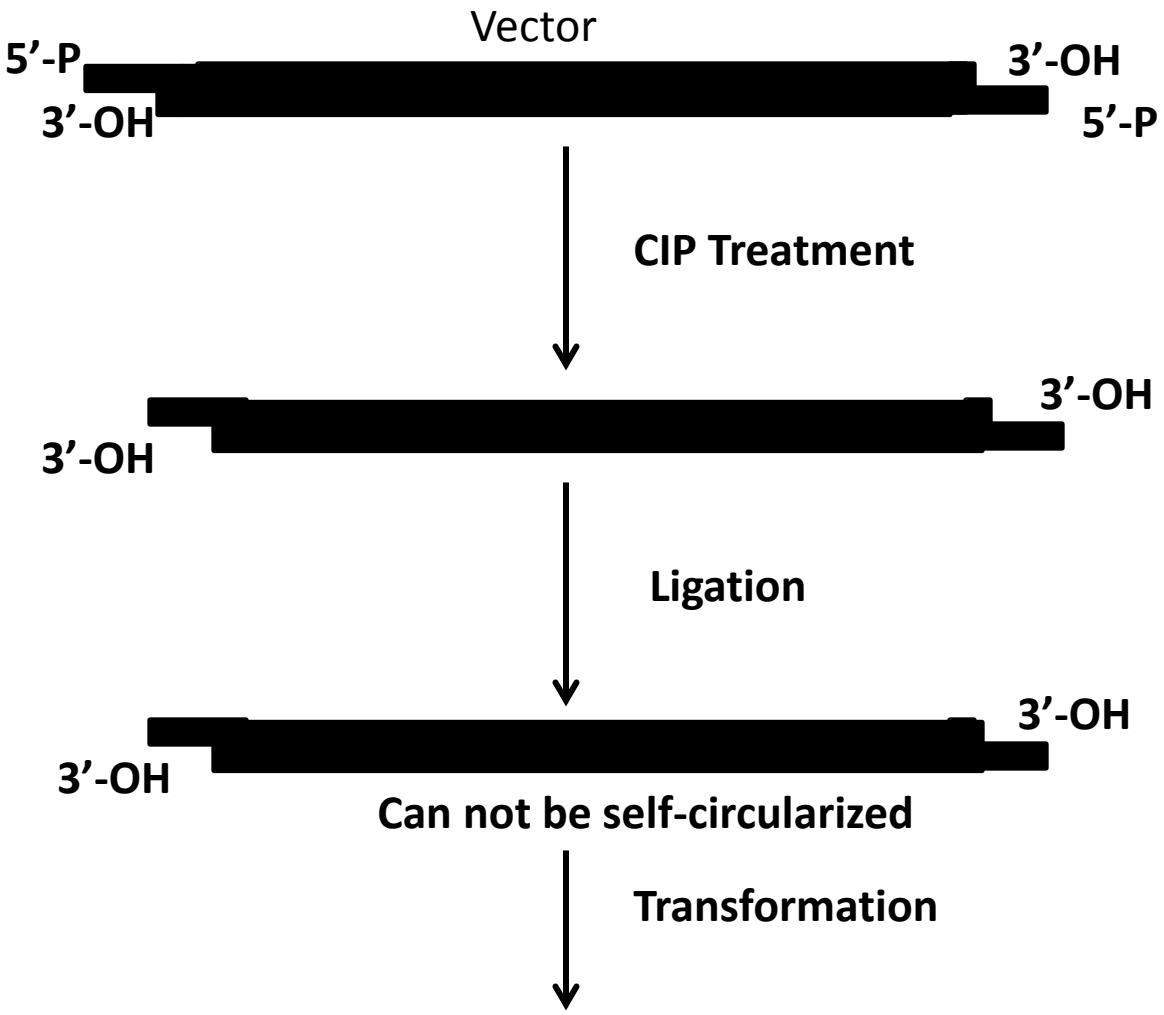
Molecular cloning, 3rd Edition

Transformation and selection after DNA ligation



Note: clone sites A and B could be blunt ends, over-hang ends, the same or different

The function of calf intestinal phosphatase (CIP)



Because the transformation efficiencies of linear DNA are very low, the backgrounds with empty-vectors are decreased radically.

Molecular cloning, 3rd edition

Choosing proper competent cells for transformation

Subcloning efficiency DH5α chemical competent E. Coli:

1 X 10^6 CFU/µg supercoiled DNA

One shot Stbl3 chemical competent E. Coli:

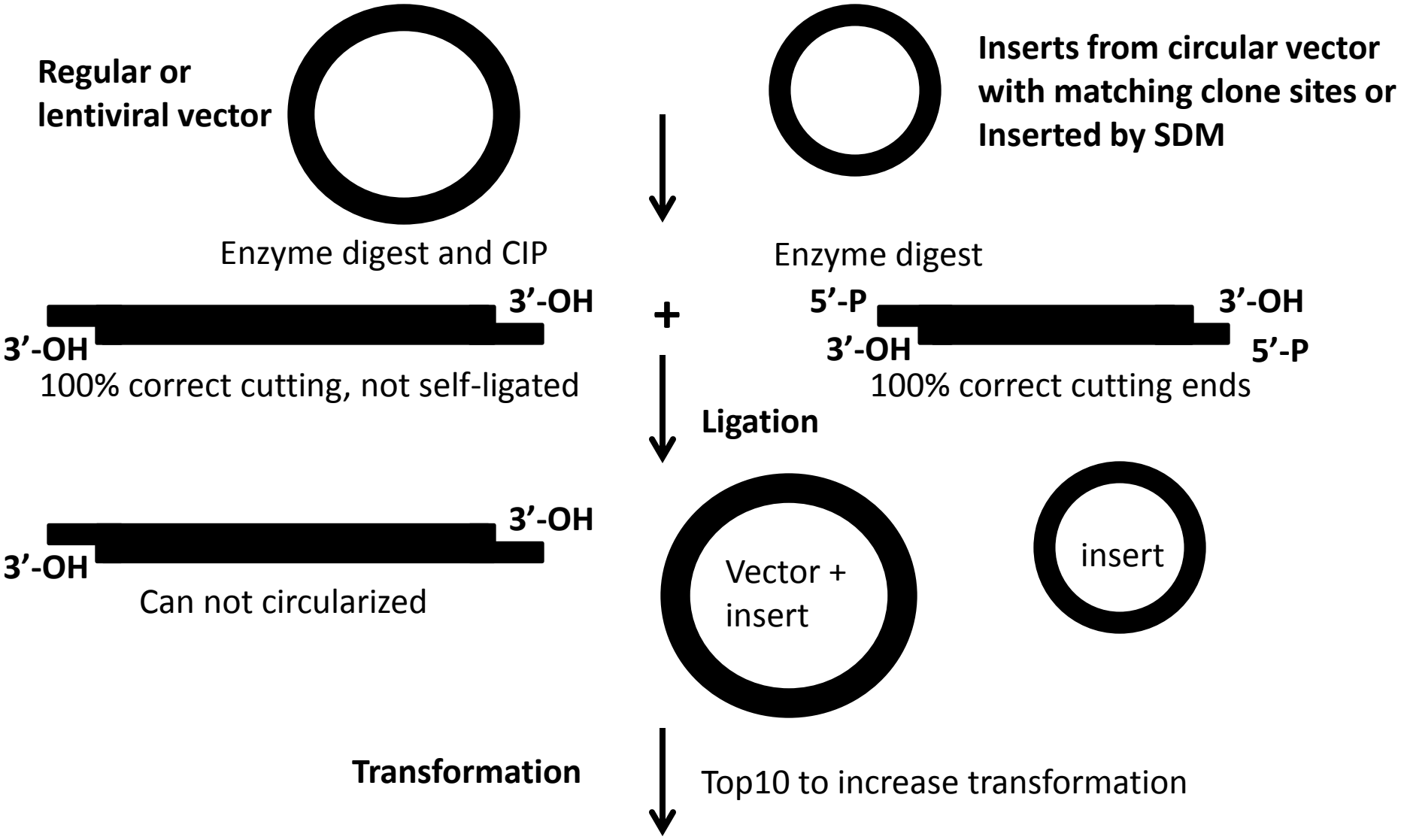
1 X 10^8 CFU/µg supercoiled DNA

One shot Top10 chemical competent E. Coli:

1 X 10^9 CFU/µg supercoiled DNA

Invitrogen (Life Technologies)

Theoretical design of combinatorial strategy



Suggestions and predictions for molecular cloning with CIP-treated vectors

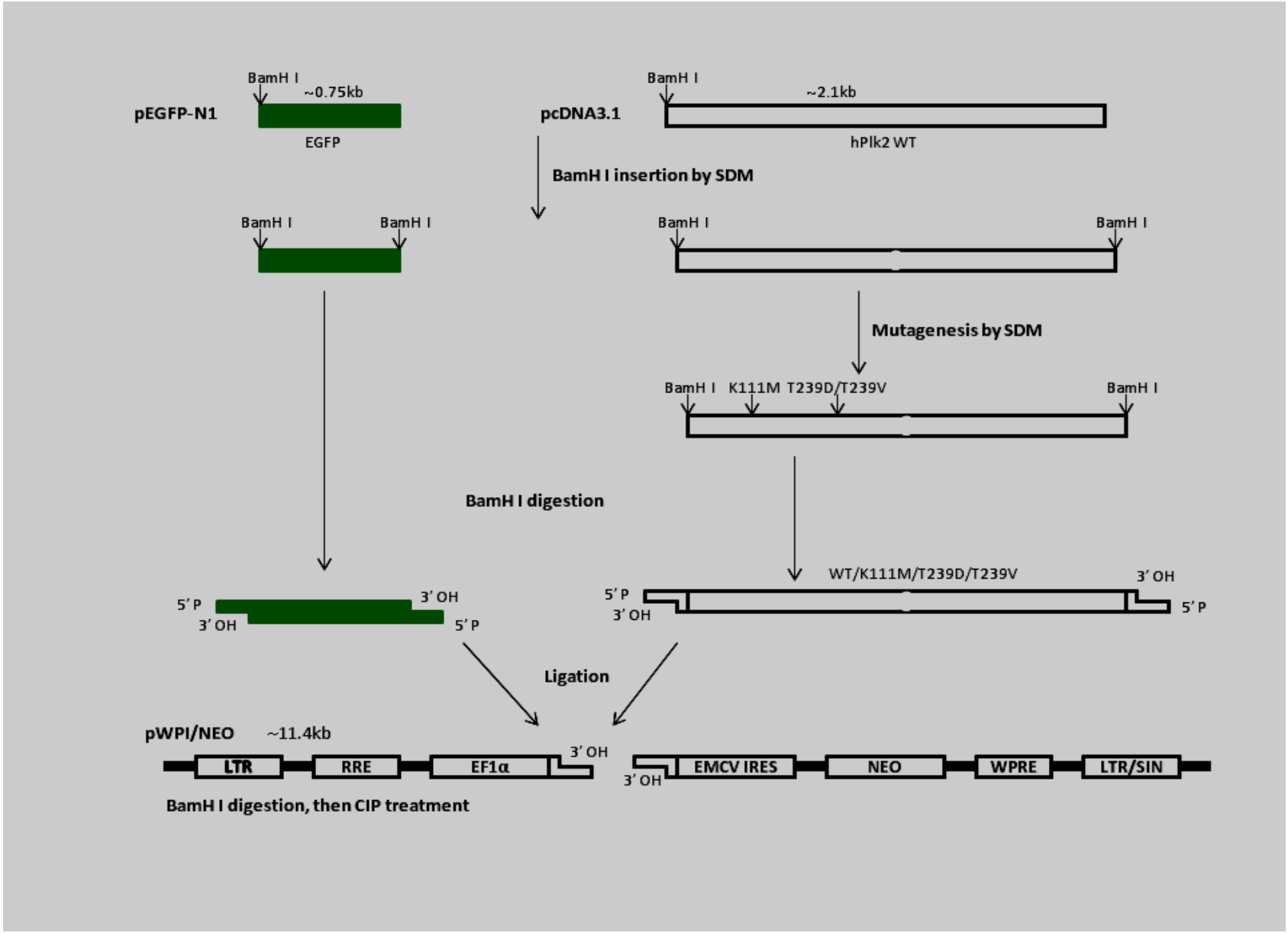
Clone sites	Sizes (kb)	Methods for clone sites	Transformation host	No. of colonies	Positive clones
Blunt sites	**Small (vector<5, insert<1.5)**	**Existed/Klenow or T4 DNA Polymerase**	**Top10**	**Dozens#/**a few	**About 50%**
	large (vector>5, insert>1.5)	**Existed**	**Top10**	**A few to dozens**	**About 50%**
Different over-hang sites	**Small (vector<5, insert<1.5)**	**PCR*/SDM**	**Top10/DH5α**	**Dozens/hundreds or more#** A few/dozens	**Nearly 100%**
	Large (vector>5, insert>1.5)	**SDM**	**Top10**	**Dozens to hundreds**	**Nearly 100%**
One over-hang site	**Small (vector<5, insert<1.5)**	**PCR*/SDM**	**Top10/DH5α**	**Dozens/hundreds or more#** A few/dozens	**About 50%**
	Large (vector>5, insert>1.5)	**SDM**	**Top10**	**Dozens to hundreds**	**About 50%**

Notes: # Data in boldfaces are obtained from existed clone sites and Top10 cell transformations.

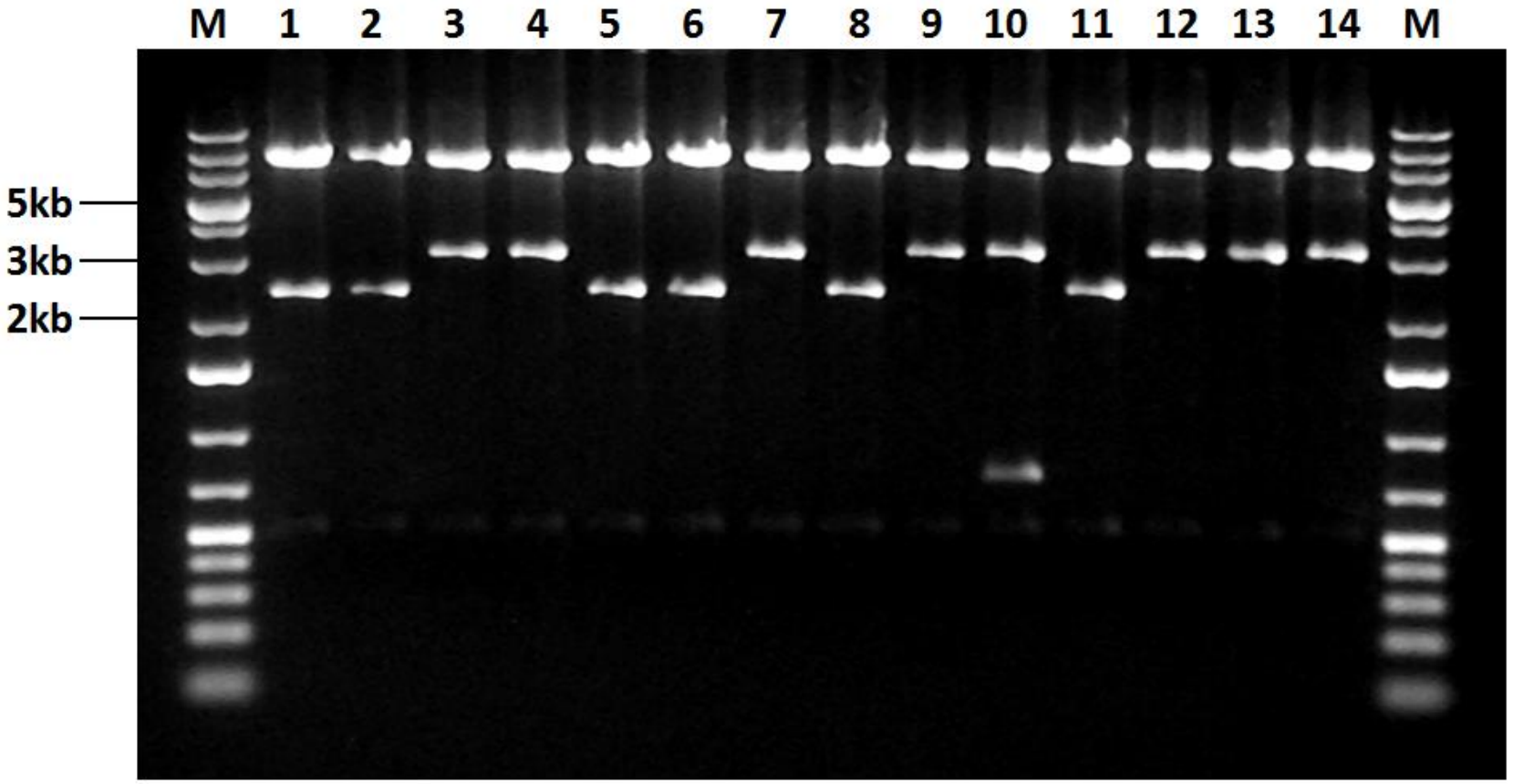
Gang Zhang, American Journal of Biomedical Research, 2013

Part II: Demonstration of combinatorial strategy with a unique BamH I clone site for lentiviral vector cloning

Scheme of clone pWPI/hPlk2/Neo and pWPI/EGFP/Neo with BamH I site

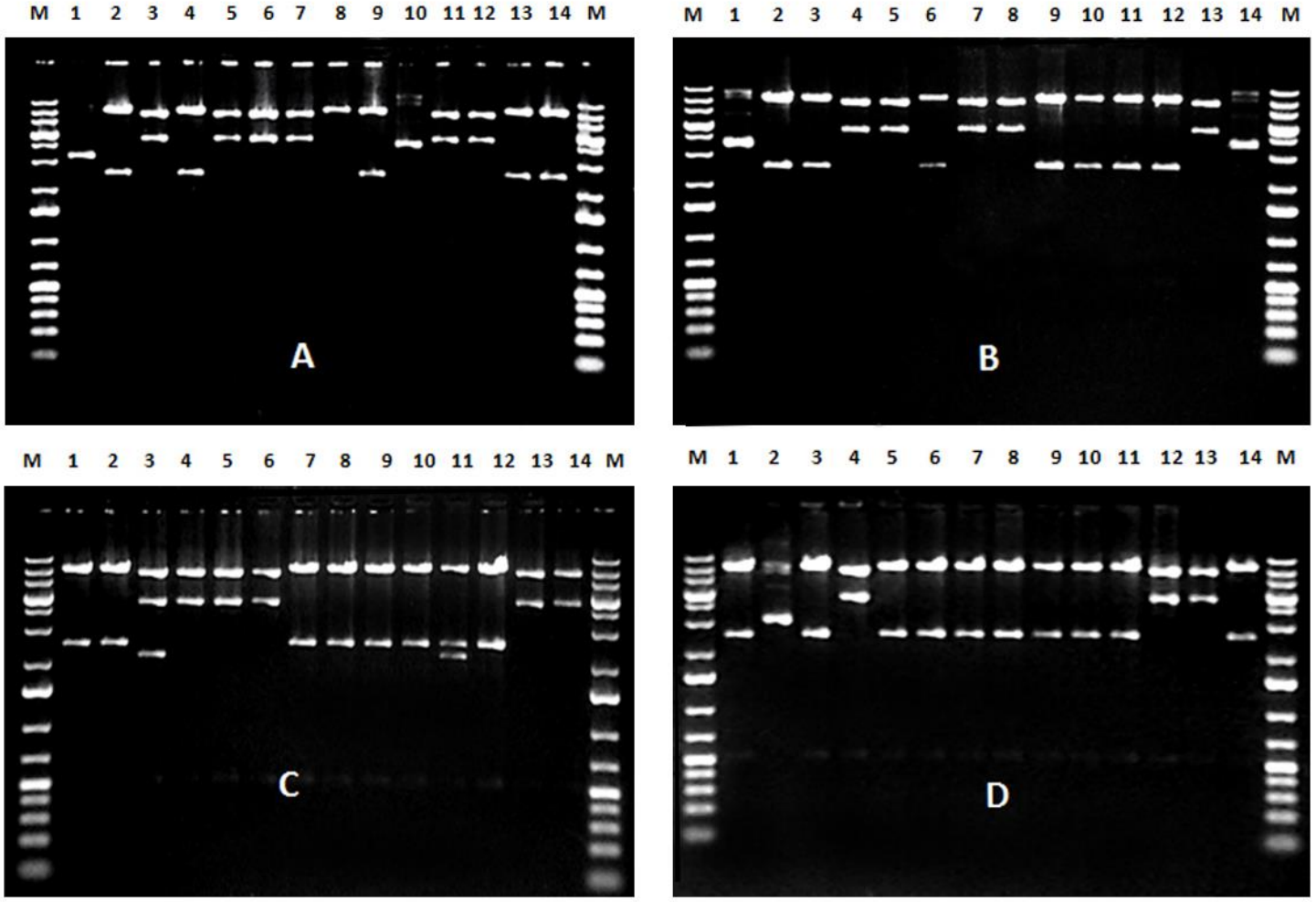


Identification of pWPI/EGFP/Neo digested by Not I (n=1)



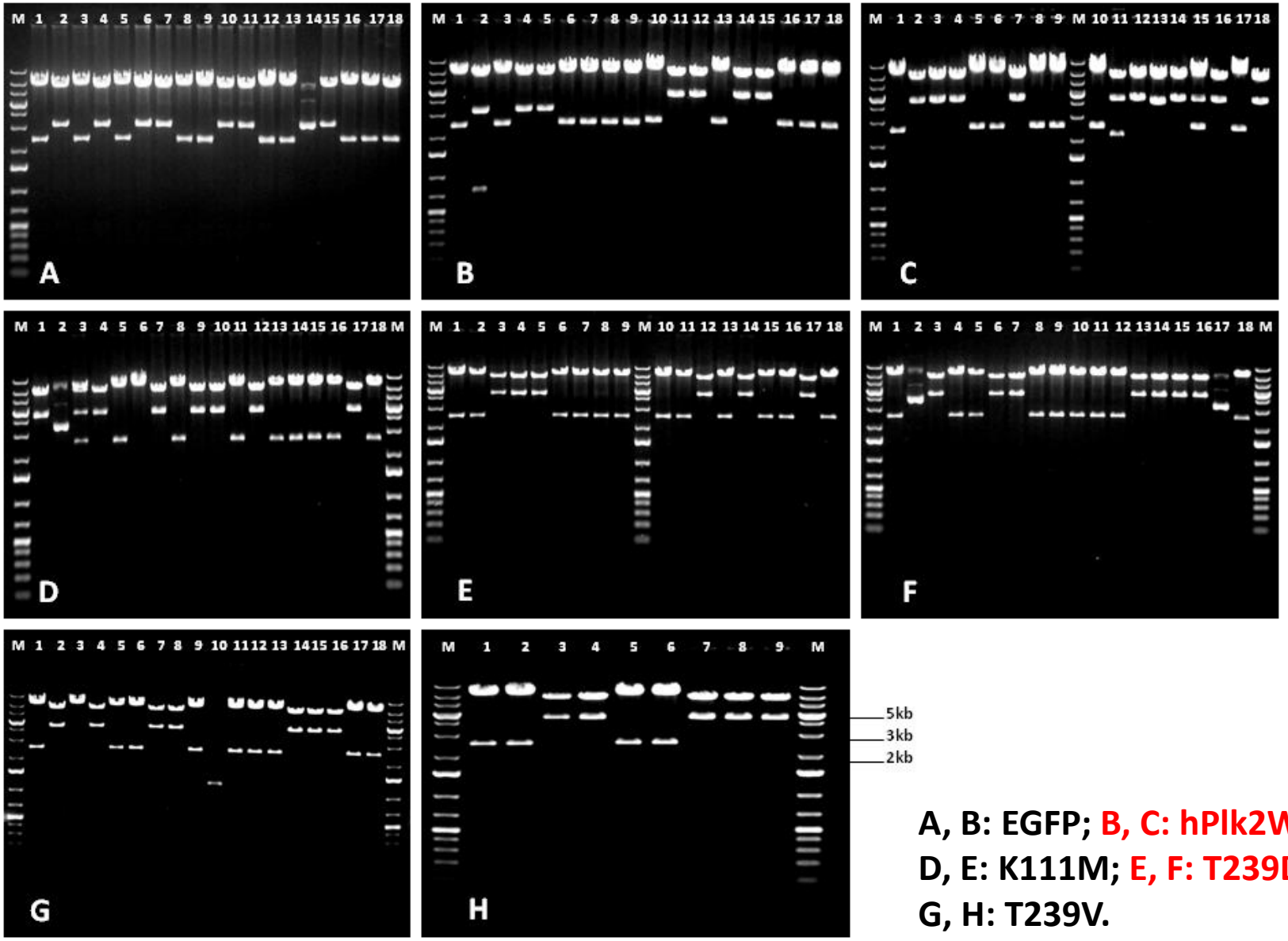
Positive clones: 3, 4, 7, 9, 12, 13, 14; Negative clones: 1, 2, 5, 6, 8, 11; Clone 10 with 2 copies of insert

Identification of pWPI/hPlk2/Neo digested by Not I (n=1)



A: WT, 2, 4, 9, 13, 14 were positive; B: K111M, 2, 3, 6, 9, 10, 11, 12, were Positive; C: T239D, 1, 2, 7, 8, 9, 10, 12, were positive; D: T239V, 1, 3, 5, 6, 7, 8, 9, 10, 11, 14, were positive.

Identification of pWPI/hPlk2 WT and mutants and pWPI/EGFP (n=3)



A, B: EGFP; B, C: hPlk2WT;
D, E: K111M; E, F: T239D;
G, H: T239V.

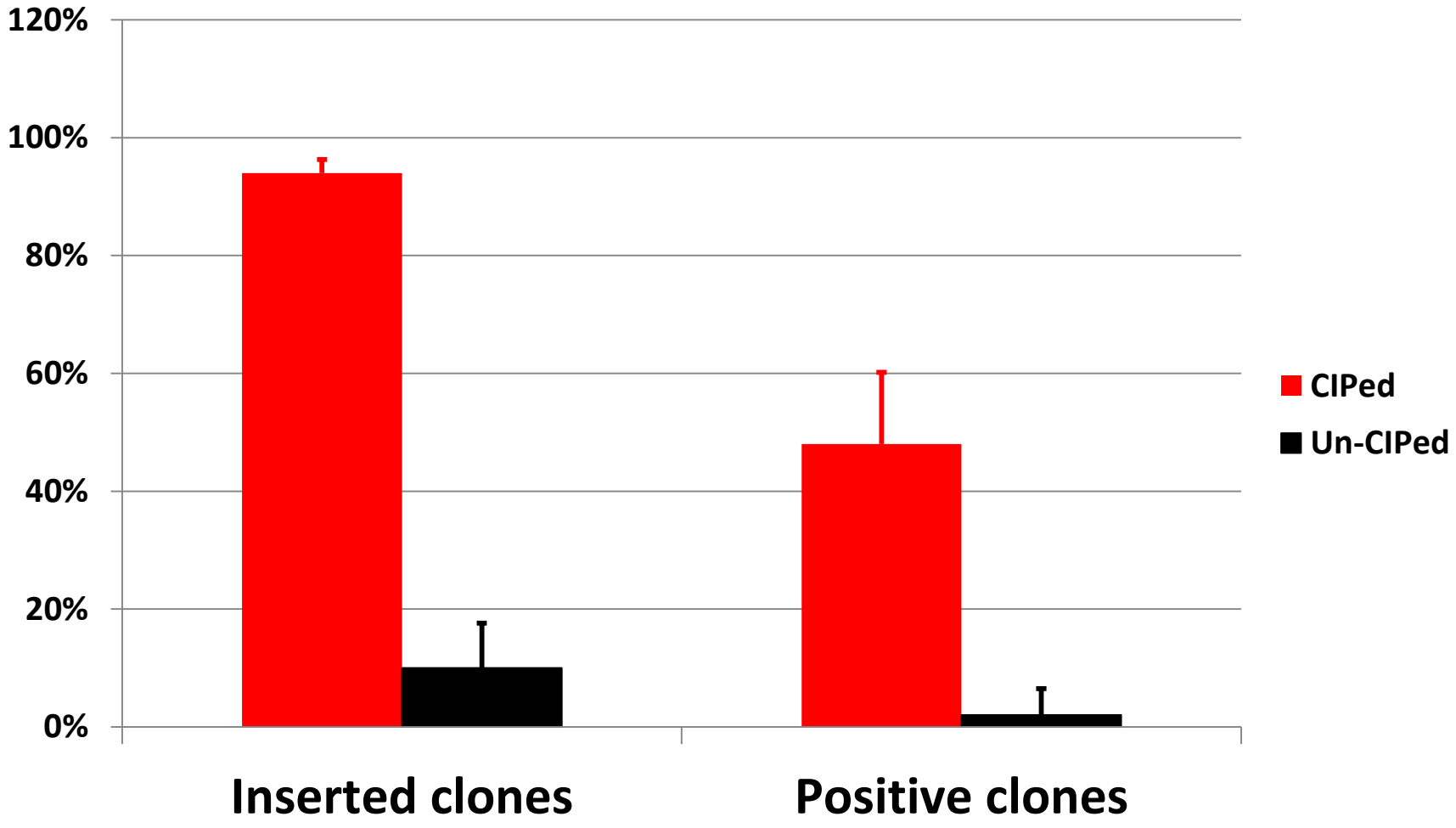
Statistical analysis of Cloning efficiencies of LVs with CIP-treated vectors (n=4)

Vector	Hosts of transformation	Total No. of transformed clones	Total No. of identified clones	Percentage of inserted vectors (Mean±SD)	Percentage of Correct-oriented inserts (Mean±SD)
EGFP	Top10	149±100 (n=4)	41 (n=4)	97%±5.5%[a](40)	37%±12.4% (16)
hPlk2 WT	Top10	123±108 (n=4)	41 (n=4)	95%±10.5% (38)	43%±16.6% (17)
K111M	Top10	123±88 (n=4)	41 (n=4)	91%±10.9% (37)	52%±21.2% (21)
T239D	Top10	126±78 (n=4)	41 (n=4)	95%±6.4%[a](39)	54%±9.8%[a] (22)
T239V	Top10	98±60 (n=4)	41 (n=4)	93%±5.2% (38)	54%±12.8% (23)

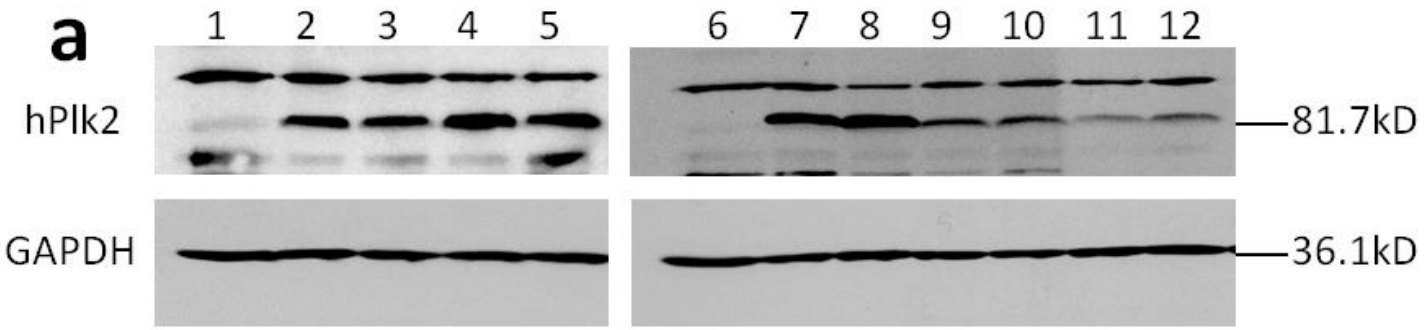
Cloning efficiencies of LVs with non-CIP-treated vectors (n=5)

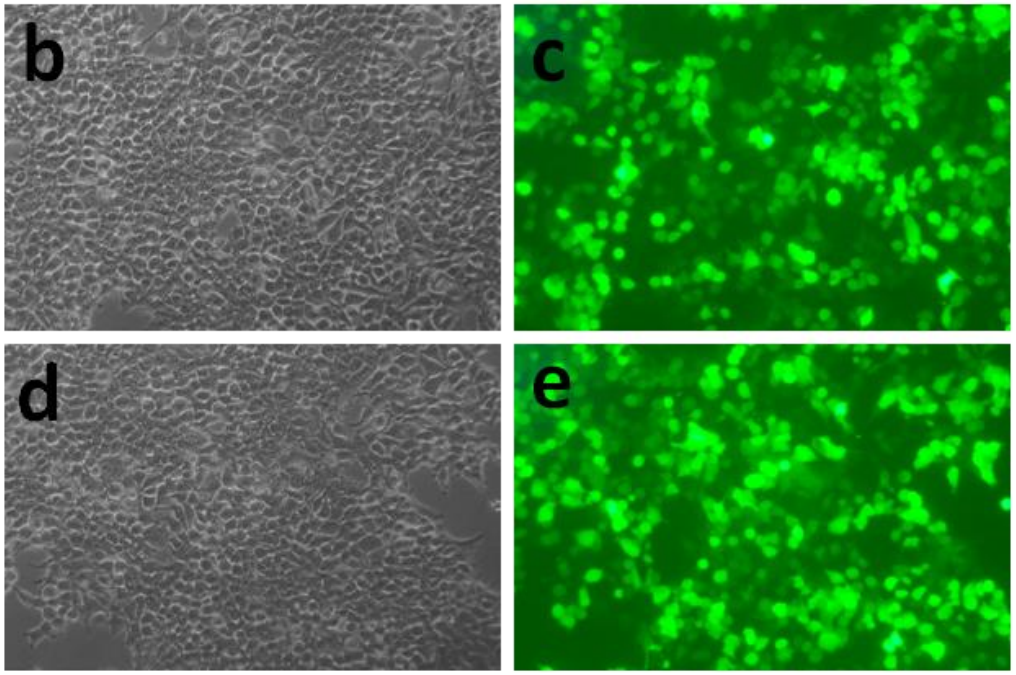
Vector	Hosts of transformation	Total No. of identified colonies	Percentage of inserted vectors	Percentage of Corrected-oriented inserts
EGFP	Top10	10	0% (0/10)	0% (0/10)
hPlk2 WT	Top10	10	10% (1/10)	0% (0/10)
K111M	Top10	10	10% (1/10)	0% (0/10)
T239D	Top10	10	0% (0/10)	0% (0/10)
T239V	Top10	10	30% (3/10)	10% (1/10)
Total			10%	2%

Cloning efficiencies of LVs with CIP-treated and un-CIP-treated vectors with BamH I site



Transient expression of hPlk2 Wt and mutants and EGFP in 293T cells





Vector sizes: ~13kb

1, 6: 293T cells;
2, 3, 4, 5: hPlk2Wt;
7, 8: K111M;
9, 10: T239D;
11, 12: T239V;
c, e: EGFP

Zhang & Tandon, Sci. Rep., 2012, 2: 415

Gang Zhang* & Anurag Tandon. Quantitative assessment on the cloning efficiencies of lentiviral transfer vectors with a unique clone site. ***Scientific Reports*****,** 2012, 2: 415

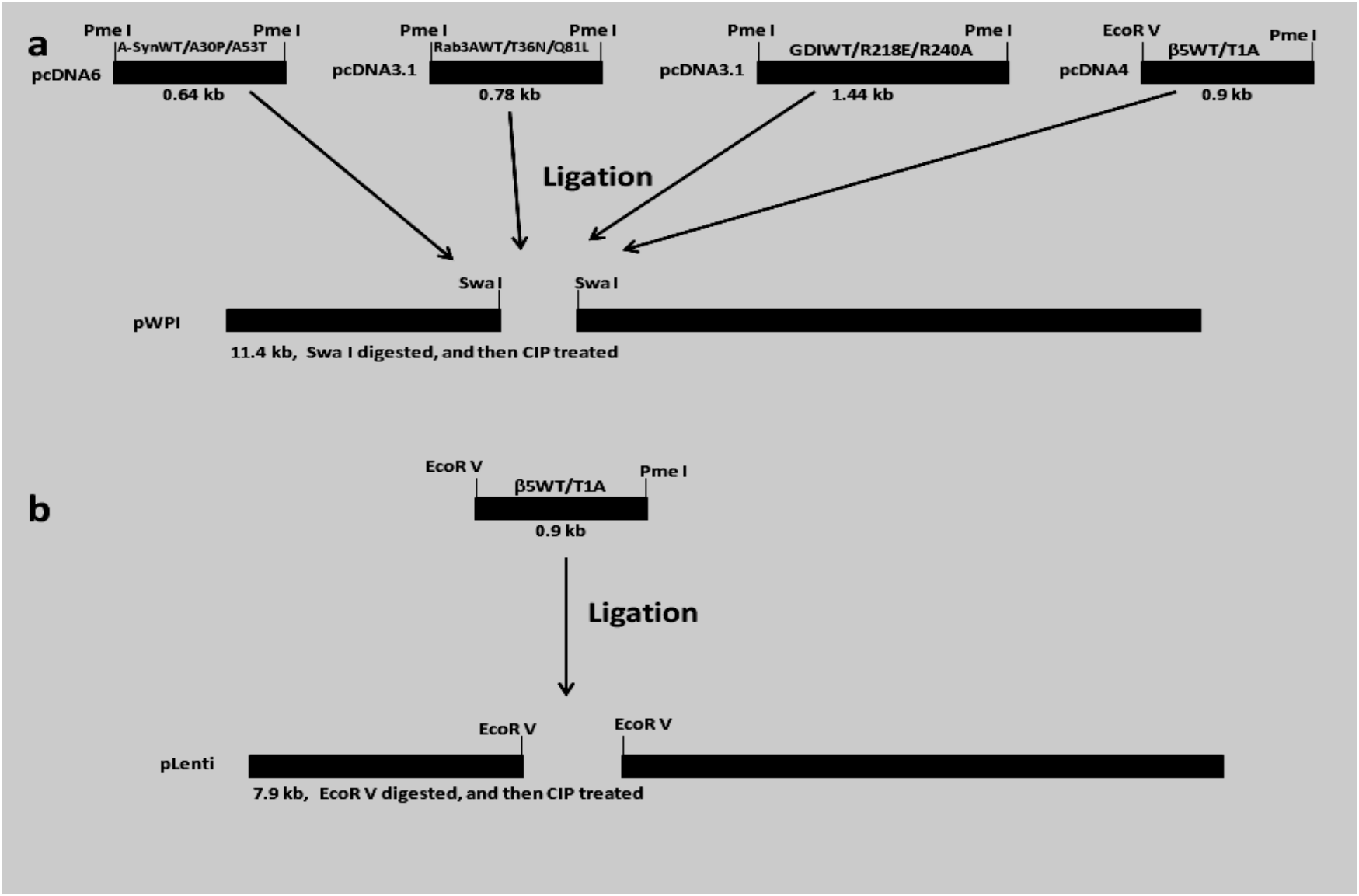
This paper is ranked #1 published on the same topic since the publication by **Isabelle Cooper-BioMedUpdater** (http://wipimd.com/?&sttflpg=23c42b52a62e87fabdf578517544b43ca5d50aa8f00f8029)

Ranked #1 in Concept-"Clone" by Scicombinator (http://www.scicombinator.com/concepts/clone/articles)

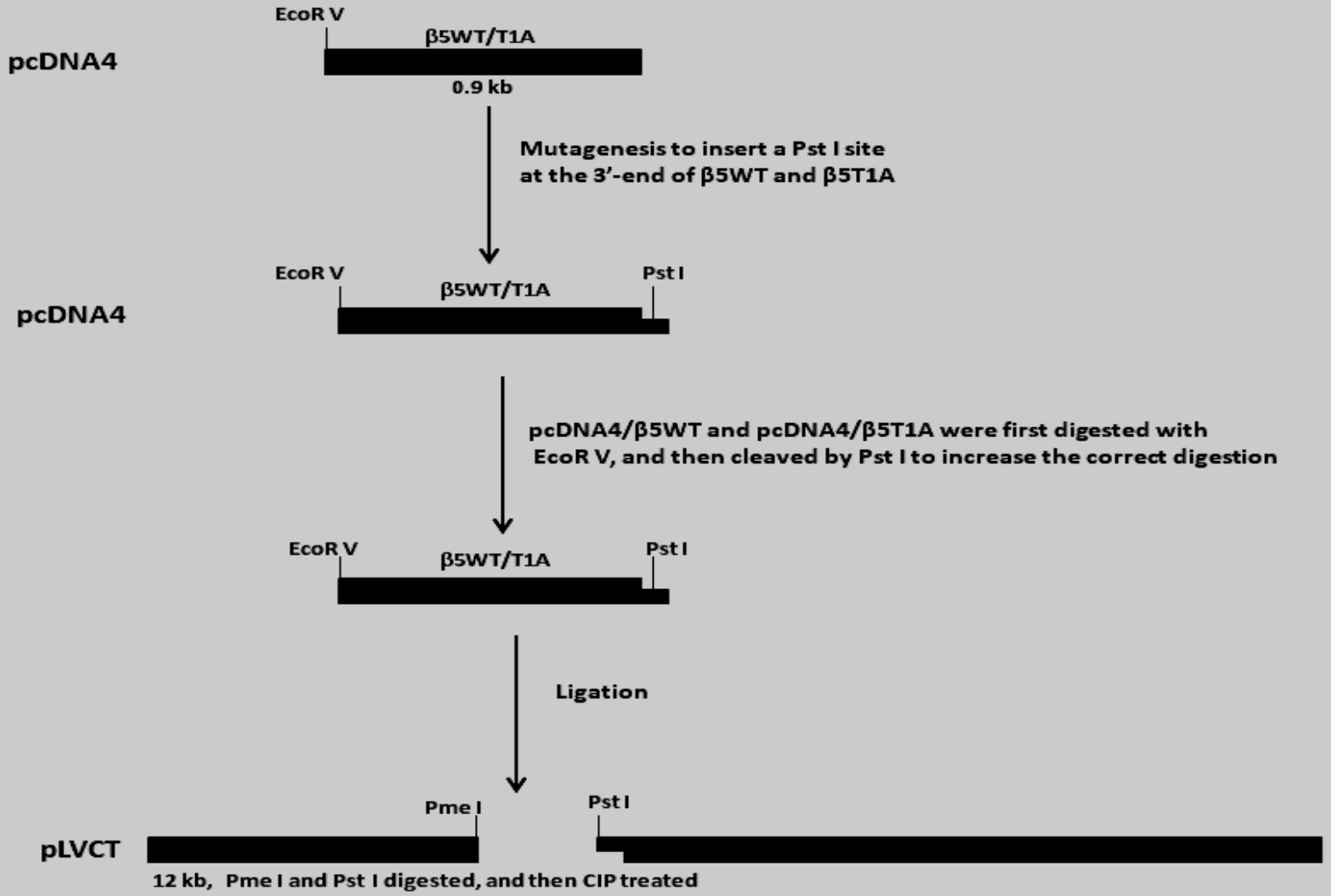
Ranked #1 in Concept–"viral vector" by Scicombinator (http://www.scicombinator.com/concepts/viral-vector/articles)

Part III: General examples for different vector cloning with various clone sites

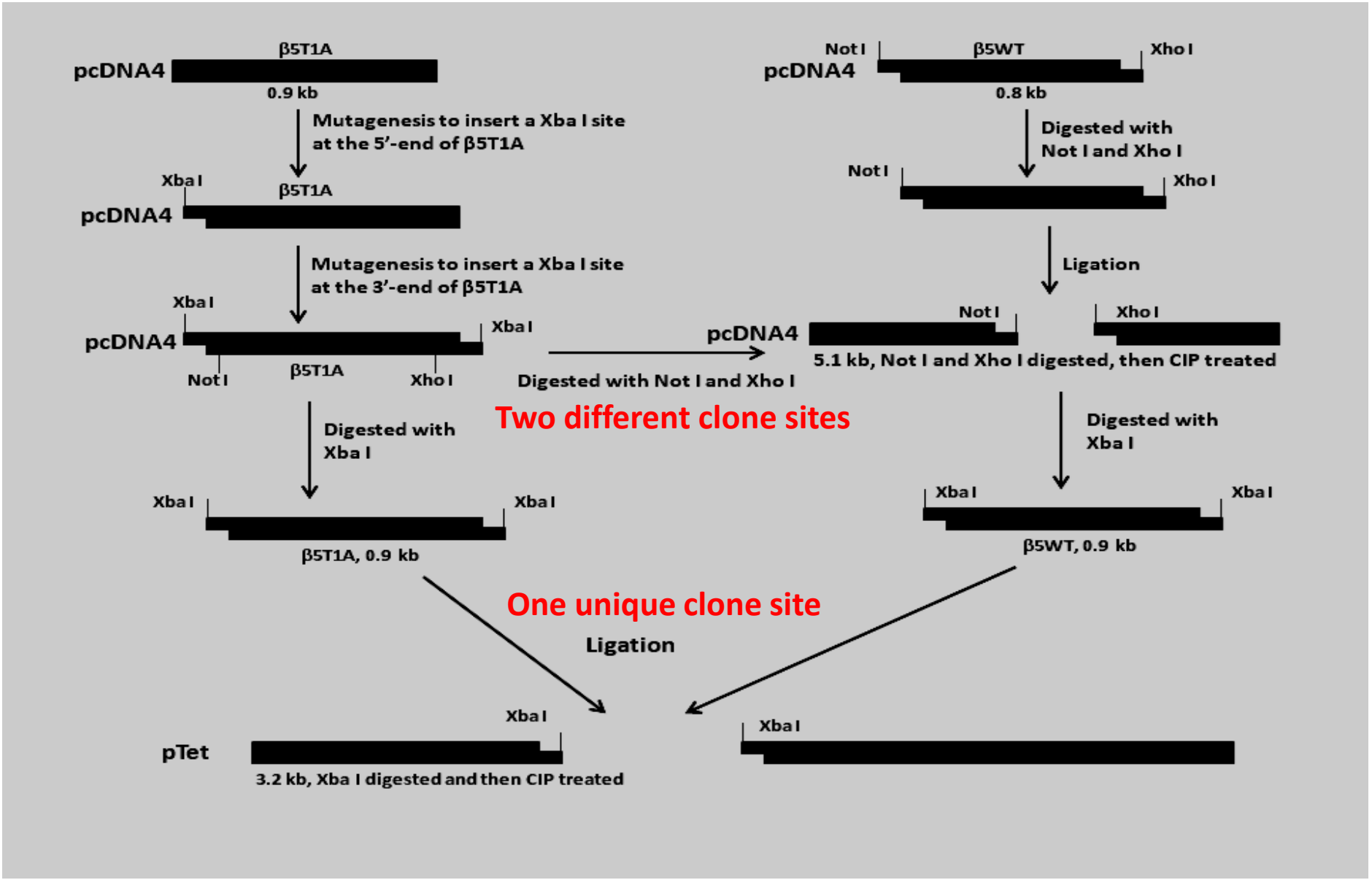
Scheme of cloning LVs with blunt clone sites (Swa I, EcoR V, Pme I)



Scheme of cloning pLVCT LVs with one blunt site and another overhang Pst I site



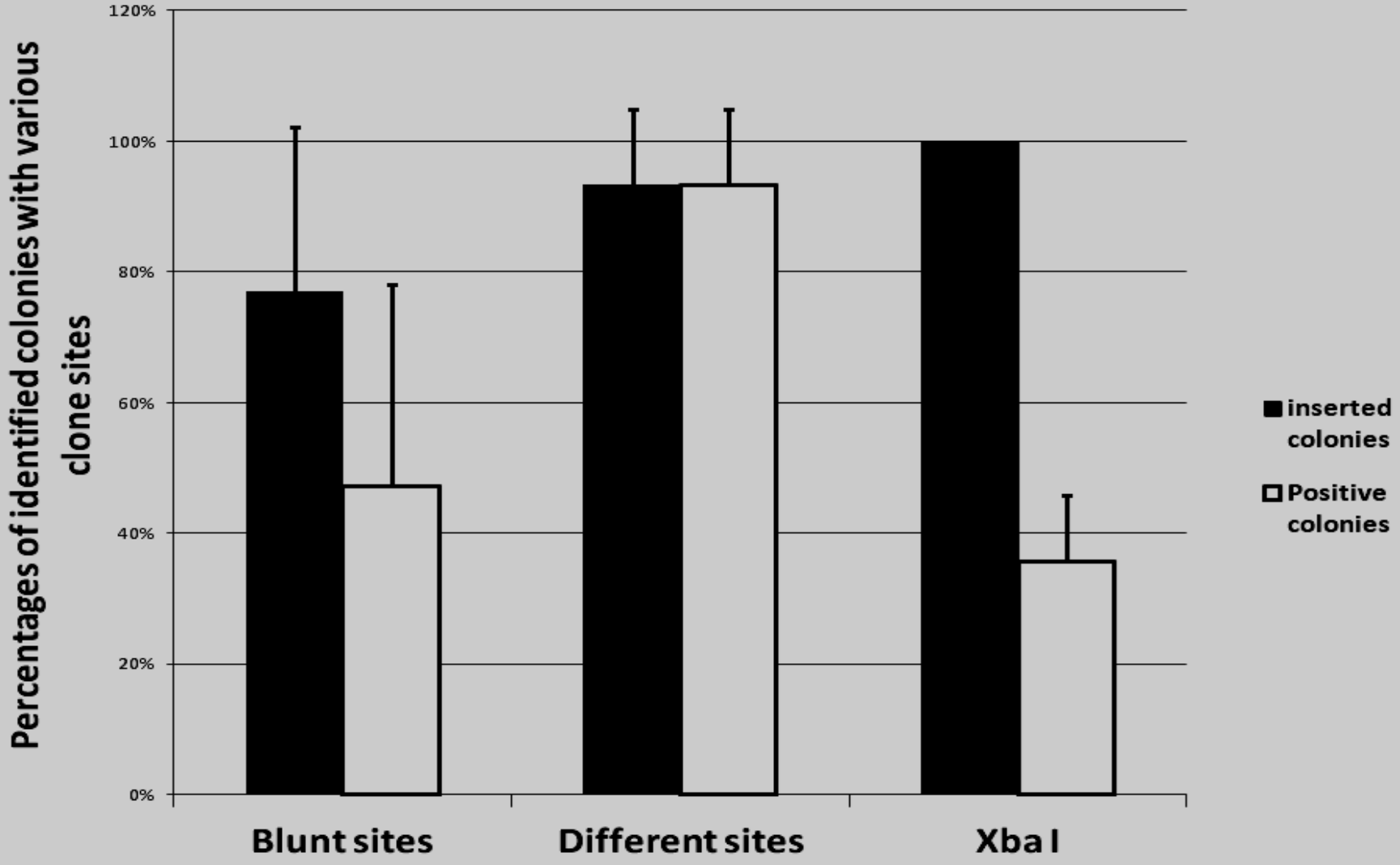
Scheme of cloning different vectors with two different overhang sites and one Xba I site



Cloning efficiencies of different vectors with various clone sites

Vector & clone sites	Inserts & clone sites	Transformed colonies	Inserted colonies	Positive colonies
pWPI (Swa I)	α-Syn-WT (Pme I)	13 (n=1)	3 (75%)	1 (25%)
pWPI (Swa I)	α-Syn-A30P (Pme I)	7 (n=1)	4 (100%)	3 (75%)
pWPI (Swa I)	α-Syn-A53T (Pme I)	10 (n=1)	1 (25%)	1 (25%)
pWPI (Swa I)	Rab-WT (Pme I)	2 (n=1)	2 (100%)	2 (100%)
pWPI (Swa I)	Rab-T36N (Pme I)	14 (n=1)	8 (80%)	2 (20%)
pWPI (Swa I)	Rab-Q (Pme I)	11 (n=1)	4 (100%)	3 (75%)
pWPI (Swa I)	GDI-WT (Pme I)	13 (n=1)	4 (66.7%)	3 (50%)
pWPI (Swa I)	GDI-R218E (Pme I)	7 (n=1)	2 (40%)	1 (20%)
pWPI (Swa I)	GDI-R (Pme I)	10 (n=1)	4 (100%)	1 (25%)
pWPI (Swa I)	β5-WT (EcoR V, Pme I)	20 (n=1)	2 (100%)	2 (100%)
pWPI (Swa I)	β5-T (EcoR V, Pme I)	2 (n=1)	1 (50%)	1 (50%)
pLenti (EcoR V)	β5-WT (EcoR V, Pme I)	12 (n=1)	6 (75%)	3 (37.5%)
pLenti (EcoR V)	β5-T (EcoR V, Pme I)	13 (n=1)	7 (87.5%)	1 (12.5%)
pLVCT (Pme I, Pst I)	β5-WT (EcoR V, Pst I)	~300 (n=1)	4 (80%)	4 (80%)
pLVCT (Pme I, Pst I)	β5-T (EcoR V, Pst I)	~100 (n=1)	5 (100%)	5 (100%)
pcDNA4 (Not I, Xho I)	β5-WT (Not I, Xho I)	~500 (n=1)	8 (100%)	8 (100%)
pTet (Xba I)	β5-WT (Xba I)	~1000 (n=1)	14 (100%)	4 (28.6%)
pTet (Xba I)	β5-T1A (Xba I)	~1000 (n=1)	14 (100%)	6 (42.9%)

Statistical analysis of cloning efficiencies of different vectors with various clone sites



Zhang & Tandon, American Journal of Biomedical Research, 2013

Conclusions

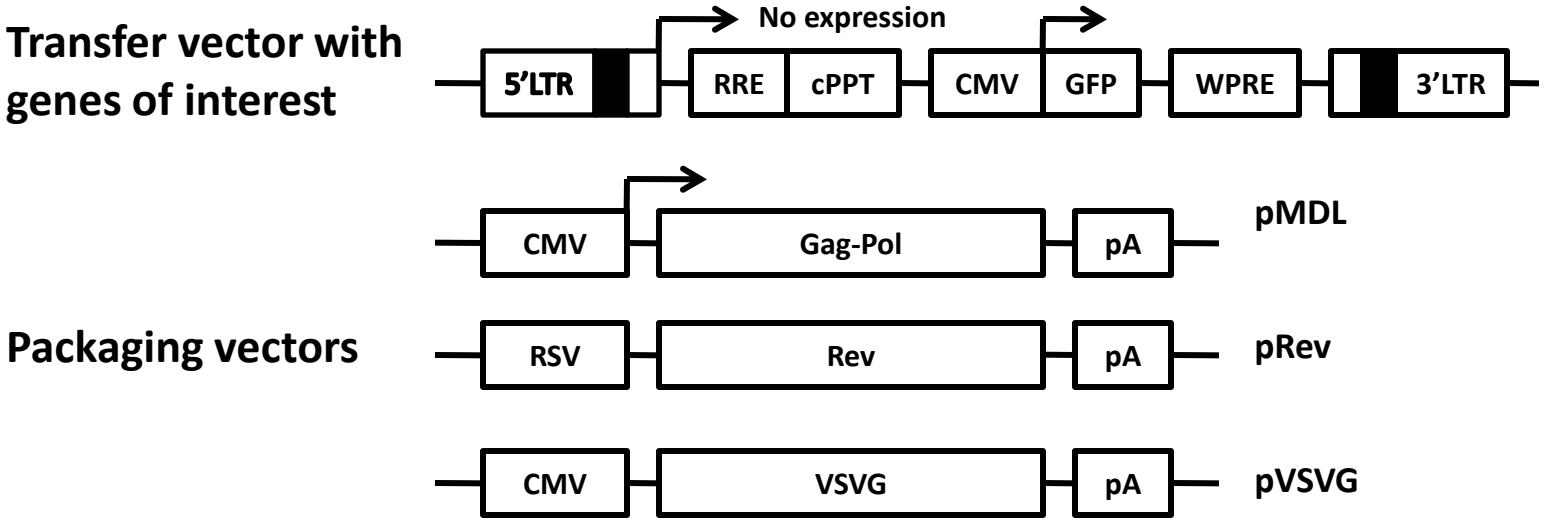
Clone sites	Positive clones
Two different clone sites	Nearly 100%
The same clone site/blunt sites	About 50%

Therefore, with our "Combinatorial strategy", almost all the plasmid vectors could be successfully cloned by "One ligation, One transformation, and 2 to 3 minipreps".

This is the quantitative law of recombinant DNA with our method.

Part IV: Lentiviral titration, and expression in mammalian cells

Scheme of The third generation lentiviral vector system



Gag-Pol precursor protein is for **integrase**, **reverse transcriptase** and **structural proteins.** I**ntegrase and reverse transcriptase** are involved in **infection**. **Rev** interacts with a **cis-acting element** which enhances export of genomic transcripts. **VSVG** is for **envelope membrane,** and lets the viral particles to transduce **a broad range of cell types**. **Deletion of the promoter-enhancer region in the 3'LTR** (long terminal repeats) is an important safety feature, because during reverse transcription the **proviral 5'LTR is copied from the 3'LTR**, thus transferring the deletion to the 5'LTR. The deleted 5'LTR is **transcriptionally inactive**, preventing subsequent **viral replication and mobilization in the transduced cells**.

Tiscornia et al., Nature Protocols, 2006

The advantages of the third generation lentiviral vectors

1. LVs can transduce slowly dividing cells, and non-dividing terminally differentiated cells;
2. Transgenes delivered by LVs are more resistant to transcriptional silencing;
3. Suitable for various ubiquitous or tissue-specific promoters;
4. Appropriate safety by self-inactivation;
5. Transgene expression in the targeted cells is driven solely by internal promoters;
6. Usable viral titers for many lentiviral systems.

Naldini et al., Science, 1996; Cui et al., Blood, 2002; Lois et al, Science, 2002

The disadvantages of the third generation lentiviral vectors

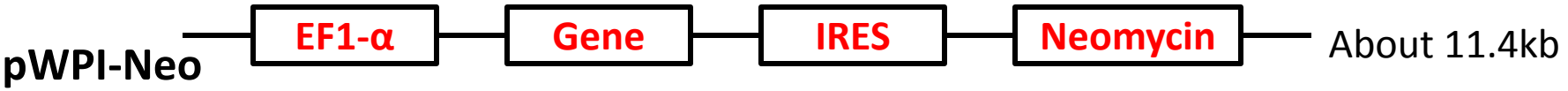
1. Lentiviral vectors are self-inactivated by the deleting of 3’-LTR region, therefore, they can not be replicated in host cells. For each lentiviral vector, the titer is solely dependent on the transfection step;

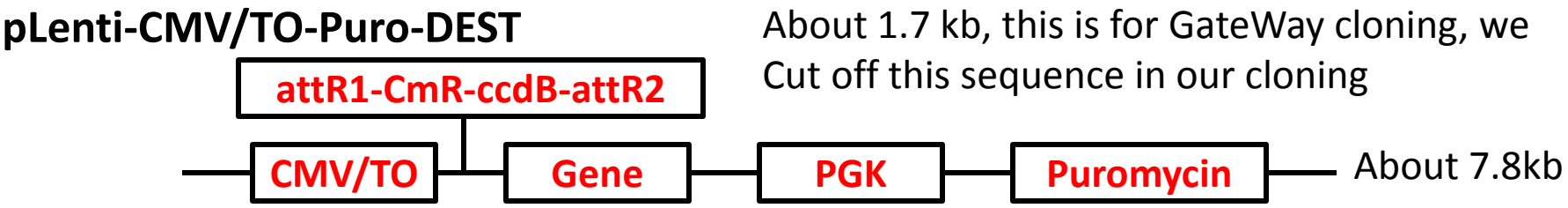
2. Only the host cells co-transfected with all the four vectors, can produce lentiviral particles for infection;

3. To make efficient lentiviral transduction, good tissue culture and transfection techniques are very important, such as lipofectamine transfection.

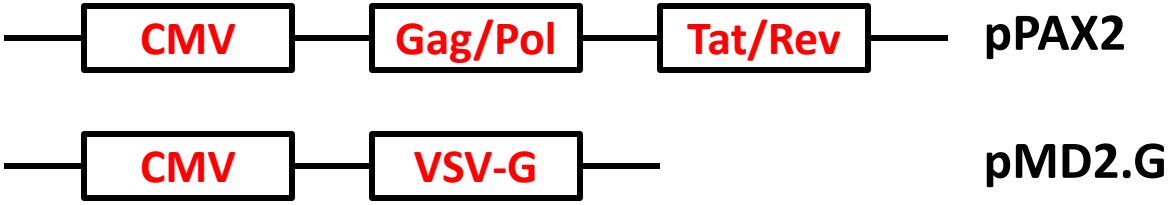
Lentiviral vector system in our research

Transfer vectors:





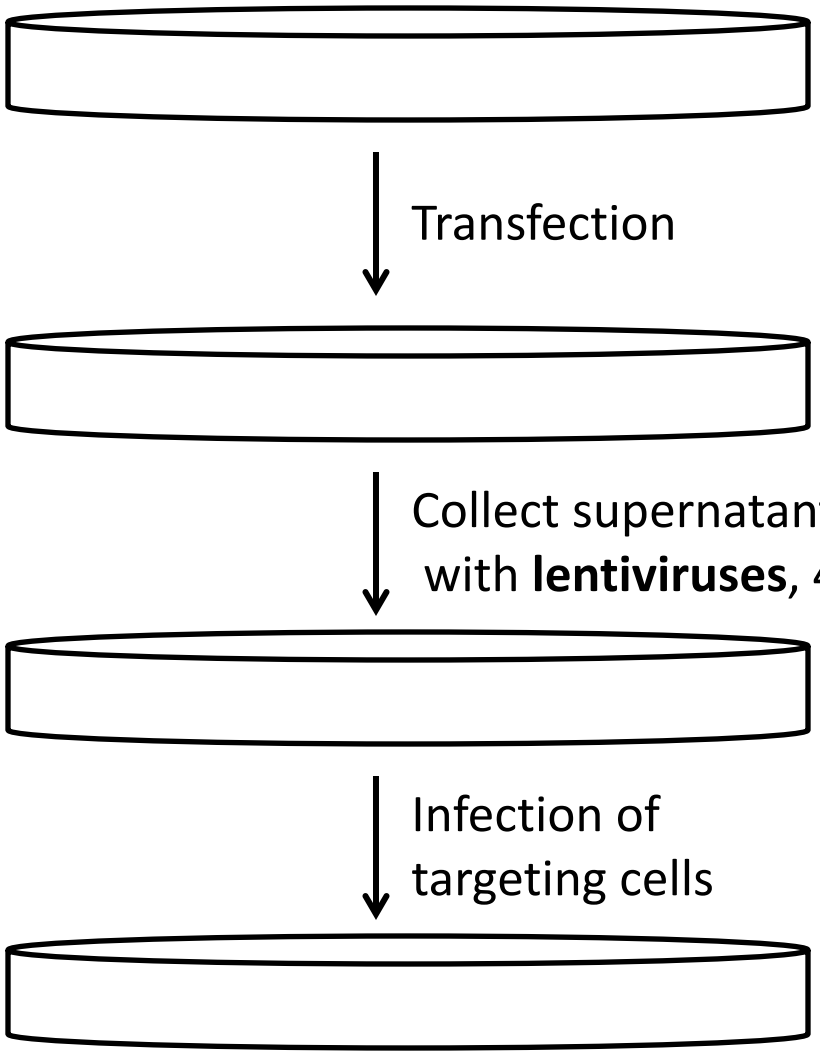
Packaging vectors:



In order to get sufficient titers, we used the third generation of transfer vectors and the second generation of packaging vectors to produce lentiviruses.

Campeau et al., PLoS One, 2009

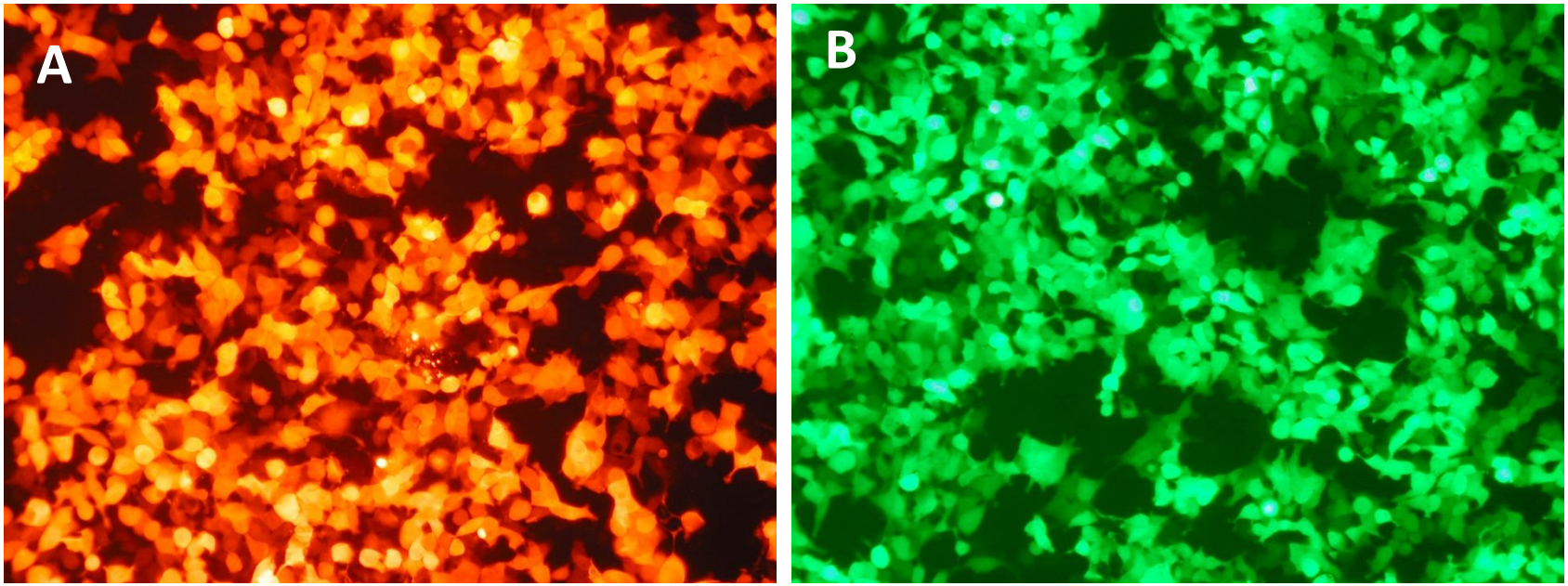
Working procedure of lentiviral transduction:



293T cells in DMEM + 10% FBS
Co-transfected with **lentiviral transfer vectors, pM2D and pPAX2 packaging vectors** by Lipofectamine

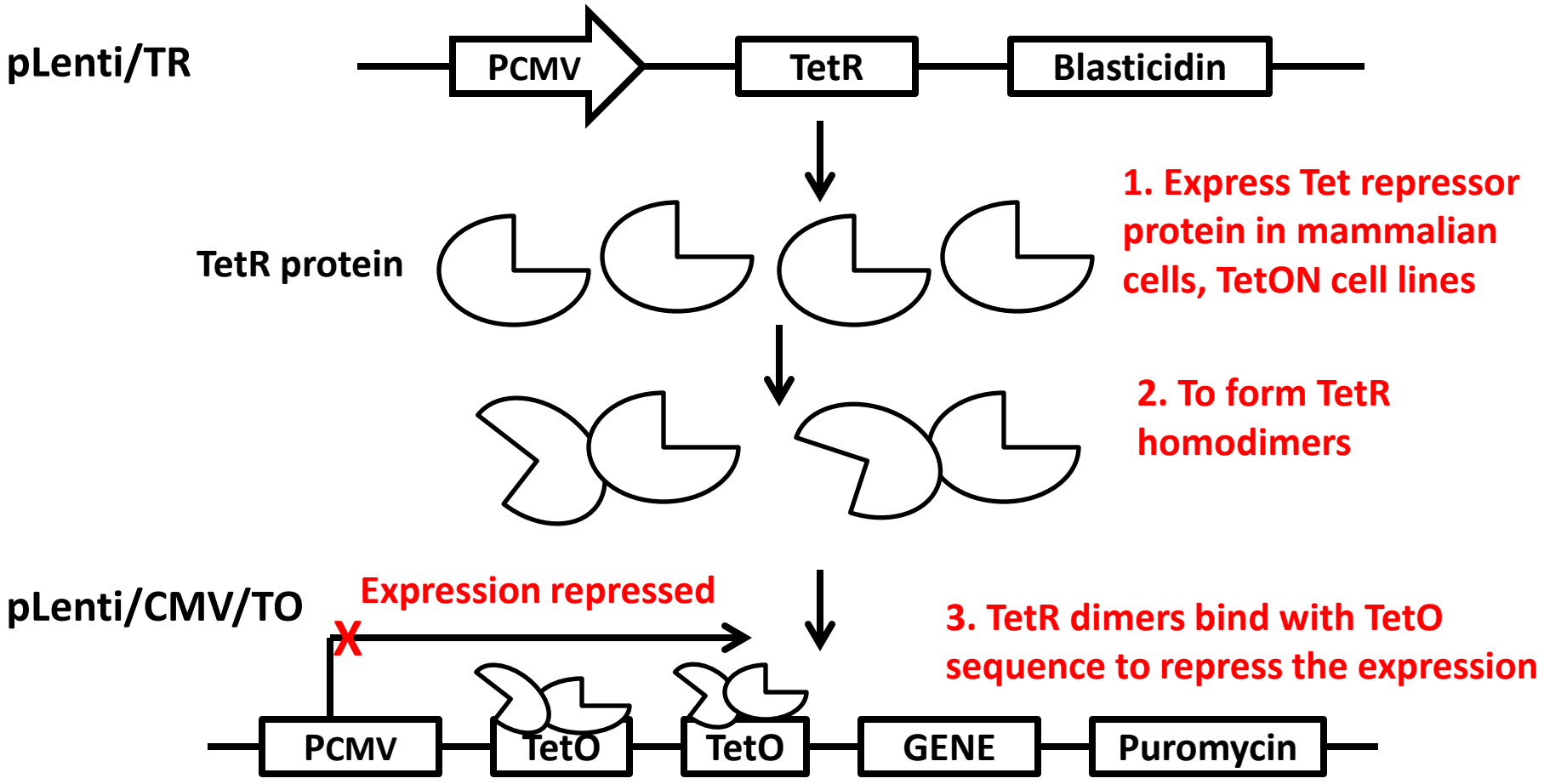
293, SHSY5Y, NSC, BV-2, etc.

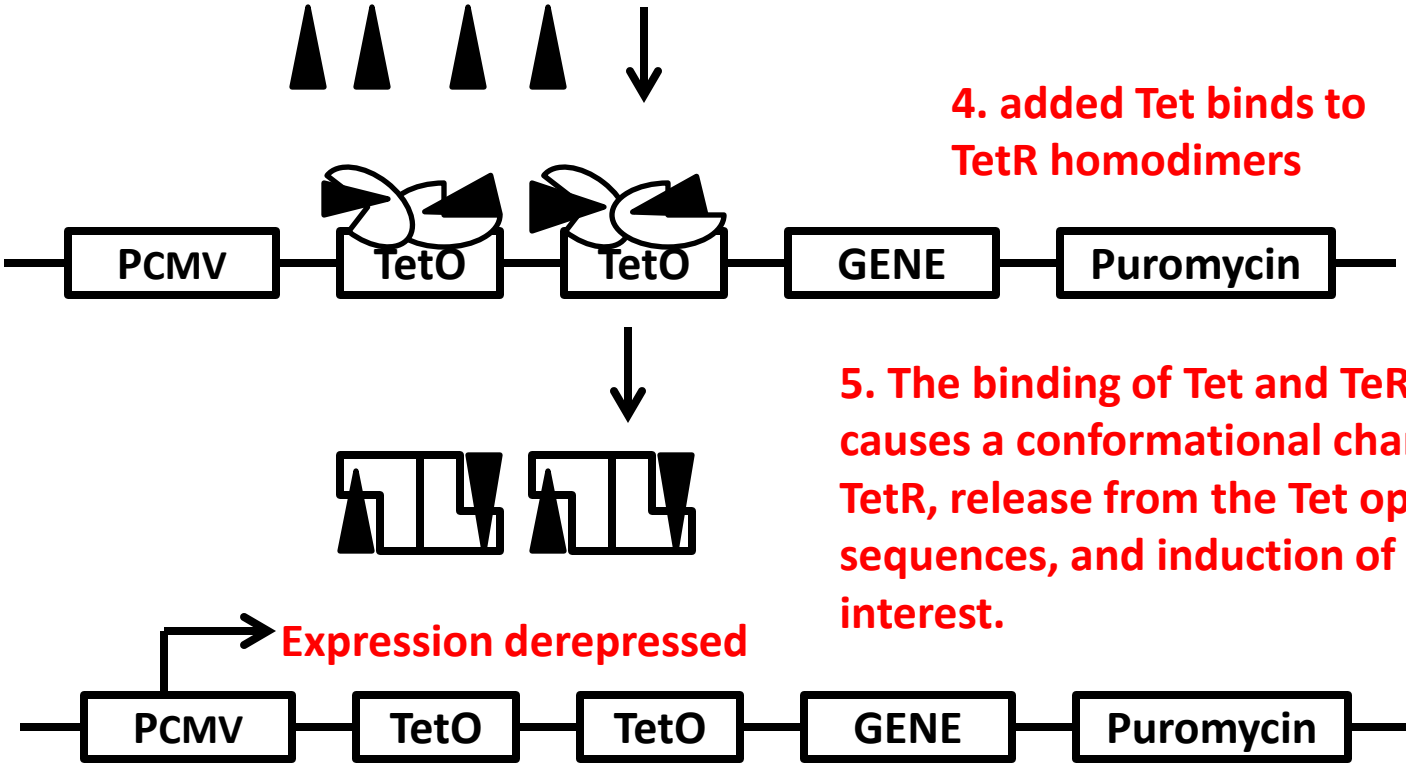
Selection
2 weeks
→ **Stable cell lines with transgenes**



A: Lipo-transfection of 293 cells with CMV-DsRed plasmid;
B: Lipo-transfection of 293 cells with EF1α-EGFP plasmid.

The mechanism of tetracycline-regulated expression system





Modified from Invitrogen

My lentiviral transductin and expression work contributed to the following papers:

1. N. Visanji, S. Wislet-Gendebien, L. Oschipok, **G. Zhang,** I. Aubert, P. Fraser, A. Tandon. The effect of S129 phosphorylation on the interaction of alpha-synuclein with synaptic and cellular membranes. **The Journal of Biological Chemistry,** 2011, 286: 35863-35873.

2. Robert HC Chen, Sabine Wislet-Gendebien, Filsy Samuel, Naomi P Visanji, **Gang Zhang,** Marsilio D, Tanmmy Langman, Paul E Fraser, and Anurag Tandon. Alpha-synuclein membrane association is regulated by the Rab3a recycling machinery and presynaptic activity. **The Journal of Biological Chemistry, 2013, (Selected as the Journal of Biological Chemistry "Paper of the Week".**

3. Cheryl A D'Souza, Melanie Dyllick-Brenzinger, **Gang Zhang,** Peter-Michael Kloetzel, Anurag Tandon. A genetic model of proteasome inhibition by conditional expression of a catalytically inactive Beta5 subunit. (In preparation).

My PH.D thesis work on mouse cloning and oocyte maturation work and publications (microinjection, confocal microscopy, tissue and embryo culture, surgeris):

1. **Gang Zhang,** Qingyuan Sun, Dayuan Chen. In vitro development of mouse somatic nuclear transfer embryos: Effects of donor cell passages and electrofusion. **Zygote,** 2008, 16: 223~7
2. **Gang Zhang,** Qingyuan Sun, Dayuan Chen. Effects of sucrose treatment on the development of mouse nuclear transfer embryos with morula blastomeres as donors. **Zygote,** 2008, 16: 15~9
3. Kong FY*, **Zhang G***, *et al*. Transplantation of male pronucleus derived from in vitro fertilization of enucleated oocyte into parthenogenetically activated oocyte results in live offspring in mouse. **Zygote**, 2005, 13: 35~8 (*** Co-first author**)

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