Development of a tetracycline-inducible lentiviral vector with an instant regulatory system

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Abstract

Lentiviral vectors, originally derived from human immunodeficiency virus, provide highly efficient viral gene delivery vehicles. Lentiviral vectors often use a constitutive promoter to drive the expression of a therapeutic gene. To regulate the expression of a therapeutic gene, a regulatory system such as Tet-On needs to be established in the target cell lines to produce a regulatory protein, reverse Tet-responsive transcriptional activator (rtTA). The expressed rtTA binds to the tetracycline responsive element (TRE) in the promoter in response to doxycycline and activates transcription of gene of interest. A hypothesis in this study is based on the speculation that a basal leaky expression of rtTA in the bi-directional TRE vectors allows instantly inducible expression of a gene of interest and thereby avoids the time-consuming procedures for generating Tet-On cell lines. Based on this hypothesis, a novel lentiviral vector has been developed to examine an instant induction of PP2Cβ as a target gene. Three instantly inducible bicistronic lentiviral vectors [pLenti-Bi-TRE-Tet-on (V), pLenti-Bi-TRE-Tet-on-PP2Cβ WT (WT), pLenti- Bi-TRE-Tet-on-PP2Cβ MUT (MUT)] were constructed and characterised to assess the usefulness of these vectors. Transient transfection of both WT and MUT vectors into HEK293T cells showed a great induction of PP2Cβ expression upon 24 h of 1 μM doxycycline treatment. The result promises the use of these vectors as a mammalian expression plasmid with a feature of inducible target gene expression. However, viral infection studies involving lentiviral packaging and infection procedures did not show a reproducible expression of rtTA or PP2Cβ in HEK293T cells. Therefore, the inducibility of viral transduction needs to be improved for the future studies of PP2Cβ in primary cells.

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Abbreviations

A	absorbance
AD	activation domain
APS	ammonium persulfate
ATP	adenosine triphosphate
attL	Left integration attachment site
attR	Right integration attachment site
b	path length
bp	base pair
BAD	Bcl-2-associated death promoter
BSA	bovine serum albumin
CA	capsid
CAK	CKD-activating kinase
cDNA	complementary deoxyribonucleic acid
CDKs	cyclin-depend protein kinase
CFU	colony-formation unit
CIP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
Cp/Ct	crossing point
CFU	colony-formation unit
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle medium
DMSO	dimethyl sulphoxide
DNase	deocyribonuclease
dNTP	deoxyribonucleotide triphosphate
Dox	doxycycline
DTT	dithiothreitol
ε	extinction coefficient
EDTA	ethylene diamine tetra-acetic acid
FBS	fetal bovine serum
FCS	fetal calf serum
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GUSB	beta glucuronidase

HEK 293T	human embryonic kidney 293T cell line
hES	human embryonic stem cell
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
IN	integrase
IκB	NFκB inhibitory binding partner
IKK	IκB kinase
IL-1β	Interleukin-1β
JUK	Jun N-terminal kinase
kb	kilo base
LB	Luria-Bertani
LTR	long terminal repeat
LV	Lentiviral vector
M	molar
MA	membrane associated matrix
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MEKK	MAP kinase kinase kinase
MLK	mixed lineage kinase
mM	millimolar
MKK	MAPK kinase
MKKK	MKK kinase
MTK1	MAP three kinase 1
MUT	vector containing mutant PP2Cβ
NC	nuclear capsid
NCBI	national centre for biotechnology information
NFκB	nuclear factor kappa B
nm	nanometer
nM	nanomolar
nt	nucleotide
OD	optical density
PEG	polyethylene glycol
Pen/Strep	penicillin-streptomycin
PIC	pre-integration complex
PMSF	phenylmethanesulfonyl fluoride

RNAi	RNA interference
PP2Cβ	protein phosphatase type 2C isoform beta
PPMs	protein phophatase magnesium-dependent enzymes
PPM1B	PP2Cβ
PPPs	phospho-protein phosphatases
PR	protease
PTPs	protein tyrosine phosphatases
PVDF	polyvinylidene fluoride transfer membrane
qPCR	quantitative real-time PCR
RCR	replication competent recombinant
RCV	replication competent virus
RE	restriction endonuclease
RHD	Rel homology domain
RNase	ribonuclease
RIN	RNA integrity number
RRE	Rev-responsive element
RT	reverse transcription/reverse transcriptase
RT-	reverse transcriptase free reaction
RT-qPCR	reverse transcription quantitative real-time PCR
rTetR	Reverse TetR
rtTA	tetracycline-responsive transcriptional activator
SAPKs	stress signaling pathways
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIN	self-inactivating vector
SU	Surface/envelop glycoprotein
TAK1	TGFβ-activated kinase 1
TBST	Tris-buffered saline with Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
tetO	tet operator
TetR	Tet represor protein
TGFα	Trasnforming growth factor α
TGFβ	transforming growth factor β
TLR	toll-like receptor
TM	Trans membrane protein

Tm	melting temperature
TNFα	tumor necrosis factor
TRE	tetracycline-response element
TREmod	modified TRE-response element
tTA	tetracycline-controlled transactivator
U	units
μM	micromolar
UV	ultraviolet
V	vector only/no PP2Cβ control vector
VSV-G	vesicular stomatitis virus G glycoprotein
WT	vector containing wild type PP2Cβ
ψ	packaging signal

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Chapter 1: Introduction

1.1 Generation of a new vector system

Vector delivery systems are commonly used as gene transfer vehicles in gene therapy, which provide promising therapeutic treatment to revert disease phenotypes. An ideal vector system for gene therapy should be bio-safe, non-toxic, non-immunogenic, tightly regulated, efficient and stable in gene expression [2]. In addition, an efficient delivery system must easily penetrate into target cells, tissues or organs, and have strict regulatory systems in place to allow precise control of gene expression [2].

A number of vector systems including viral and non-viral vectors have been used to deliver genes of interest into target cells while providing efficient and robust expression of the transgenes. Viral vectors are considered superior to non-viral vectors because they offer broader tropism, higher transduction efficacy, greater cloning capacity, and more efficient penetration into target cells and their nuclei [3]. Most importantly, viral vectors have the ability to integrate into the host genome rather than remaining episomal, allowing long-term stable expression [4, 5]. Due to safety concerns, however, viral vectors without most of the virus-derived coding sequences have been used in gene therapy. This helps to minimize any adverse immune responses by preventing the recognition and destruction of transduced cells by cytotoxic T lymphocytes [5].

1.1.1 Lentiviral systems

Lentiviral vectors are retroviruses that are derived from human immunodeficiency virus (HIV). Pseudotyping, a process that produces viral vectors in combination with foreign viral envelope proteins, with the vesicular stomatitis virus G glycoprotein (VSV-G) allowed a broad transduction tropism of lentiviruses [6]. A unique characteristic of LV vectors is their ability to replicate in non-dividing cells via a nucleoprotein complex, called pre-integration complex (PIC), allowing the association of viral genetic material to their host genome [3, 6-10]. This feature made lentiviral vectors more appealing to researchers.

1.1.1.1 Basic biology and evolution of lentiviral vector

Generally, lentiviruses consist of two copies of RNA, a capsid (CA), a nuclear capsid (NC), a membrane associated matrix (MA), and various envelope proteins and enzymes (Table1.1 and Figure1.1). The major envelope proteins include surface/envelop glycoproteins and trans-membrane proteins (TM); while the major envelope enzymes include integrase (IN), protease (PR) and reverse transcriptase (RT). The coding region of *gag*, encodes the CA, NC and MA proteins while the *pol* and *env* genes are critical for pro-virus integration and polyadenylation, respectively. The *gag*, *pol* and *env* regions are further flanked by long terminal repeats (LTRs) and *cis*-acting sequences [3]. The two regulatory genes, *tat* and *rev*, regulate HIV gene expression transcriptionally and post-transcriptionally and are critical for viral replication. Lentiviruses also carry four accessory genes *nef*, *vif*, *vpu*, *vpr* which are dispensable for viral growth but have been shown to be required for *in vivo* replication and pathogenesis [3, 7].

Table 1.1: The function of lentivirus components.*

Name	**Function**
Capsid (CA)	*gag* gene; protect the core
Nuclear Capsid (NC)	*gag* gene; protect the genome and formation of the core
Membrane associated matrix (MA)	*gag* gene; lines envelope
Surface/envelop glycoprotein (SU)	*env* gene; Polyadenylation
Tans membrane protein (TM)	The inner component of the mature envelope
Integrase (IN)	*pol* gene; integration of the provirus
Protease (PR)	Essential for *gag* protein cleavage during maturation
Reverse transcriptase (RT)	Reverse transcribes the RNA genome
Accessory protein for HIV	*nef*, *vif*, *vpu*, *vpr*; *in vivo* replication and pathogenesis

*Table is obtained from Viginia Commonwealth University (VCU) website [11].

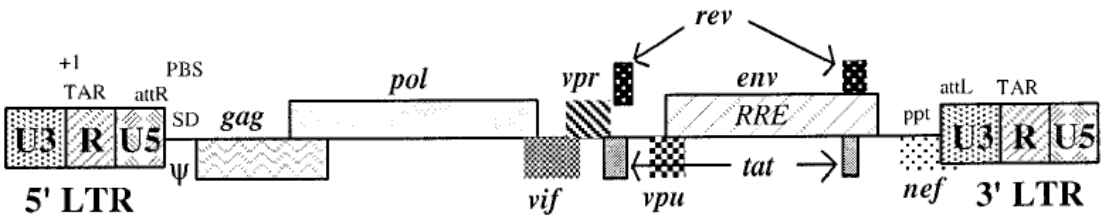

Figure 1.1: A schematic representation of HIV-1-derived vector structure. Nine of HIV genes: *gag*, *pol*, *tat*, *rev*, two regulatory genes *tat* and *rev*, and four accessory genes *nef*, *vif*, *vpu*, *vpr*. Both long terminal repeat (LTR) includes U3, R and U5 regions. att, integrase attachment site; SD, splice donor; ψ, packaging signal; ppt, polypurine tract (Figure adapted and modified from [12], permission for publishing obtained through RightsLink®)

The complexity of the lentiviral genome has been exploited by scientists for years to build a novel vector with appropriate biosafety to minimize reconstitution of the wild-type virus by recombination. To avoid the emergence of replication competent recombinants (RCR), many precautions were taken, for example, the genes required for virus packaging and replication are now expressed by two separate plasmids [5, 6].

1.1.1.2 Packaging process

The construction of a lentiviral gene delivery system requires two major components: (i) a vector genome containing the gene of interest for transfer (Figure 1.2a), and (ii) a packaging helper genome, also called a packaging plasmid, which provides necessary enzymes and protein structures to generate infectious viral particles (Figure 1.2b) [5, 7]. For biosafety concerns, the helper virus genome is devoid of the packaging signal (ψ) preventing repackaging of the virus. Upon co-transfection, the vector and packaging plasmid are introduced into a packaging cell line, commonly human embryonic kidney (HEK) 293T cells. Here, the packaging plasmid undergoes transcription and translation to produce viral structural and replication proteins (Figure 1.2c) while the vector genome is transcribed into a single-stranded RNA genome that is recognized by the packaging signal (ψ) (Figure 1.2d). Once virions are packaged and assembled inside of the packaging cell line (Figure 1.2e), they are released as viral particles containing viral vector (Figure 1.2f).

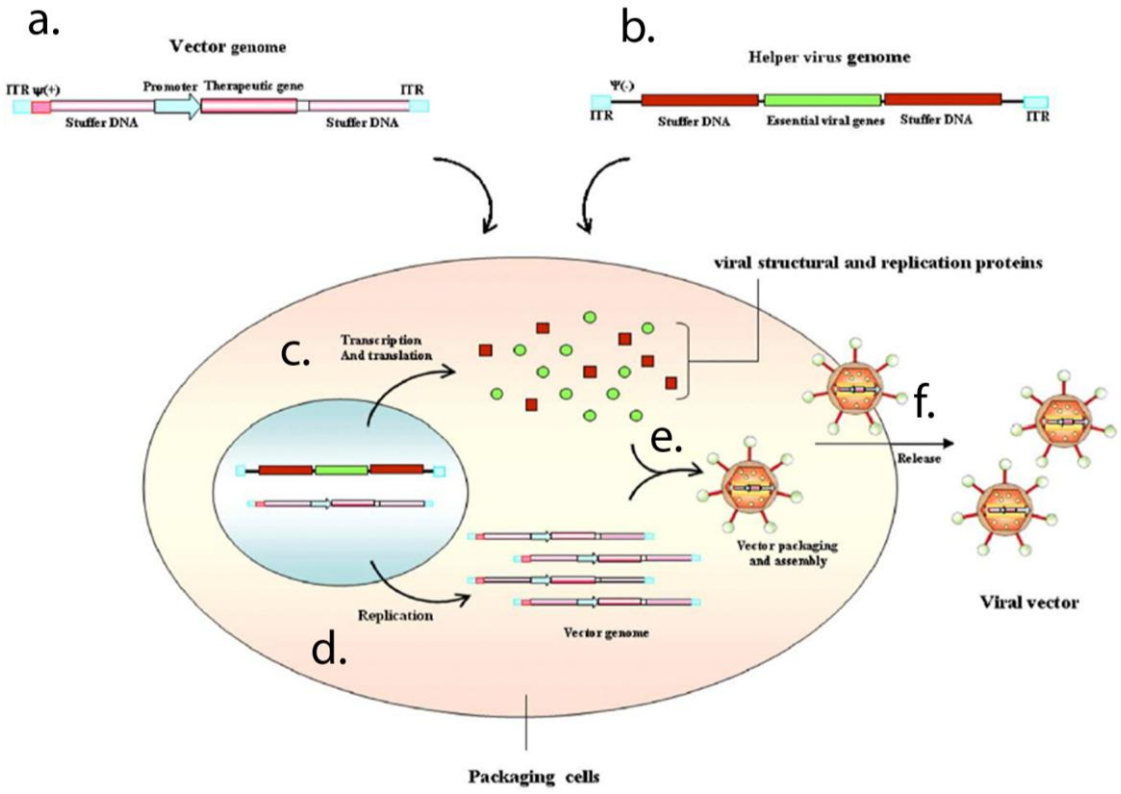

Figure 1.2: Virus packing system. Harboured within the helper viral DNA (**b**) are viral genes encoding for proteins necessary for viral genome replication (**a**) The therapeutic gene expression cassette, flanked by the inverted terminal repeats (ITR) and cis-elements which is required for genome encapsidation is contained within the vector genome. **a**: vector genome; **b**: helper virus genome; **c**: transcription and translation of virus structural and replication proteins; **d**: vector genome replicates into single stranded RNA genome; **e**: virions package and assembly; **f**: viral vectors release out of packaging cells (Figure adapted from [2], permission for publishing obtained through RightsLink®).

1.1.1.3 Development of lentiviral vectors

The first generation of HIV-derived vectors comprised of all HIV proteins (Table 1.1) apart from the envelope protein [5, 7]. Although the viral particles were produced in a separate packaging plasmid, the high content of HIV proteins could risk re-constitution of the wild-type virus. To alleviate this, second generation vectors contained only the key components *gag*, *pol*, *tat* and *rev*. Later studies suggested that *tat* is also dispensable in the packaging construct, by replacing the *tat*-dependent U3 sequence from the 5' LTR with strong heterologous promoter sequences to hinder the transcription of vector genomic RNA in producer cells, therefore, promotes a *tat*-independent primary transcription without affecting the efficiency of the vector generation [5, 7]. Optimal

production of viral vector requires the presence of *rev* which encodes a post-transcriptional regulator, Rev-responsive element (RRE), that strictly regulates the expression of *gag* and *pol* [7, 13]. It has been shown that vectors containing Rev/RRE systems exhibit the highest yield in production [6]. The third generation of HIV-derived vectors was further stripped down to only three core HIV-1 genes, *gag*, *pol* and *rev*. In addition, the original viral genome has been further split to express *rev* from a separate construct [14]. It has been demonstrated that a split genome dramatically reduces the emergence of RCR, eliminates targeted infection of CD4+ T-lymphocytes and extends cell tropism allowing safer and broader application of therapeutic interventions [3, 6]. This third-generation vector system significantly prevents the risk of parental virus regeneration, since 60% of the genome has been completely eliminated [13].

To further improve these vector systems, the two terminal repeat (LTR) sequences have been removed to form self-inactivating (SIN) vectors. Removal of these sequences abolishes the generation of replication competent viruses (RCV). The LTR sequences are transcriptional elements containing enhancer and promoter sequences, the left integration attachment site (attL) in U3 at 3' LTR, the right integration attachment site (attR) in U5 at 5' LTR, polyadenylation signal in R, part of the polyadenylation signal, and *Tat*-interacting TAR sequence overlapping the R region (Figure 1.1) [12, 15]. SIN vectors are achieved by further deletion of viral enhancer and promoter sequences, in order to eliminate the transcriptional capacity of the viral LTR in integrated target cells, which prevents the mobilization of RCV [3, 12, 14, 16, 17].

In addition, it has been shown that *Tat trans* activation requires an intact TATA box; mutations in the TATA box dramatically reduce viral titers [15, 18]. In contrast, *Tat*-independent transcription, achieved by replacing the U3 region of 5' LTR with the cytomegalovirus (CMV) promoter, did not affect viral titer production [12, 16]. Other modifications, for example, U3 deletion and U5 substitution of the 3' LTR also exhibited a competitive transduction efficiency compared to the wild type construct [15, 18]. The *cis*-acting element guides polyadenylation of viral genomic RNA distal to the TATA box, which resides upstream of the R region in LTR (Figure 1.1); however, polyadenylation on the *cis*-acting element may lead to a decrease in efficiency [5]. Together this evidence suggests that HIV-1 derived vectors may tolerate a large or complete U3 deletion without functional loss; however, the efficiency may be reduced

to some extent. These modifications of the LV vector design provided additional safety features to the lentiviral vector system.

1.1.1.4 Advantages, limitations and concerns of lentiviral vectors

Lentiviral vectors are known to facilitate high-level transduction, broad tropism and long-term expression in a variety of cells and tissues. As one of the gene delivery systems in gene therapy, the superior advantage of lentiviral vectors is their ability to infect non-dividing cells without delivering lethal viral coding sequences. This prevents the recognition and destruction of transduced cells by cytotoxic T-lymphocytes [5]. In addition, several advancements have effectively made general safety concerns redundant. These include incorporating pseudotyping with envelope glycoproteins, elimination and reassembly in multiple attenuated split genome packaging systems as well as the development of the third generation and SIN lentiviral vectors [3, 6, 9, 19].

Despite a lentiviral vector gene delivery system being an effective tool in gene therapy, there are still some limitations. For instance, lentiviral vectors often lack convenient multiple cloning sites (MCS), increasing the difficulty of introducing transgenes into the system [3]. Although transgene expression can be readily controlled by administration of ligand of transcriptional regulators such as the antibiotic, doxycycline (Dox) in Tet-On/Off systems, regular or prolonged exposure may induce unwanted side effects such as drug resistance in clinical trials [2]. It has also been shown that the relatively safe SIN vectors which contain deletions in the TATA box, displayed a lower viral titer and were impaired in induced penetration of the target tissue and organ resulting in reduced transduction efficiency [5, 16]. Another disadvantage of these systems is that the absence of the accessory protein *vpr* and *vip* abolishes *in vivo* infection in target tissues such as liver and muscle [13]. Thus, vectors used for targeting these tissues could potentially increase the cytotoxicity and the generation of RCRs. In addition, untoward immune effects of the transcriptional activator proteins are also a major concern in lentiviral systems [2, 20, 21].

A more recent lentiviral system has been improved by incorporating regulatory switches to tightly regulate transgene expression (see Section 1.1.2). These switches are able to induce transgene expression to increase the transduction efficiency, and therefore, the

requirement of the viral vector dose is reduced for enhanced utility and biosafety of gene transfer vectors [2]. A concern still remains however, that these regulatory systems can be leaky resulting in toxicity and inefficient induction.

1.1.2 Tetracycline regulatory system

Regulatory systems of lentiviral vectors have been developed to control the expression of transgenes *in vivo* and *in vitro*. Successful gene therapy not only requires an efficient delivery vehicle, but also needs a stringent and reliable regulatory system. An ideal pharmacologically-regulated system in human gene therapy should: (1) contain an On/Off switch to allow tight regulation, (2) be activated by a highly specific orally administered small molecule that does not interfere with endogenous metabolic pathways, (3) have low basal expression and high inducibility, (4) have low immunogenicity and high bio-safety, and (5) be dose-dependent and orally bio-available [17, 22]. There are a number of regulatory systems available, including the most widely used tetracycline-controlled system.

The tetracycline-controlled system can be activated by the antibiotic tetracycline and its derivatives such as doxycycline (Dox), which is a second generation semisynthetic analog of tetracycline. Dox was developed in 1967 and has since been successfully used in clinical treatments for more than 40 years [23-26]. Dox offers several advantages over tetracycline including (1) a much easier dose schedule compared to tetracycline; (2) a reasonably long half-life; (3) being more readily absorbed when taken with food avoiding gastrointestinal upset, and (4) is generally well tolerated in the body with fewer side effects [27]. Additionally, the high lipid solubility of Dox promotes better distribution and tissue penetration compared to tetracycline, allowing potentially improved penetration of the blood-brain barrier [23, 25, 27].

Besides these clinical applications, Dox is also widely used in research applications as an inducer in tetracycline-inducible gene regulation systems, especially in the context of viral vectors. In *Escherichia coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn 10 transposon. In the absence of tetracycline/Dox, TetR dimerises and binds to tet operator (*tetO*) DNA sequences consequently blocking the transcription of this operon [24, 28, 29]. However, in the

presence of tetracycline/Dox, a conformational change in TetR leads to disassociation from *tetO* rescuing the transcriptional blockade [28]. The sequence-specific and high-affinity binding between TetR and *tetO* also provides the basis of regulation and induction for mammalian experimental systems.

The natural Tet-controlled DNA binding domain (DBD) of *E.coli* TetR is fused to the Herpes simplex virus VP16 heterologous transcriptional activation domain (AD) to form a hybrid protein, tetracycline-controlled transactivator (tTA). In the absence of Dox, the fusion of VP16 AD converts the TetR from a transcriptional repressor to a transcriptional activator activating expression of the transgene (Figure 1.3A). The reverse TetR (rTetR) was developed by four amino acid changes in TetR. In the presence of Dox, rTetR binds to *tetO* to form the reverse tetracycline-responsive transcriptional activator (rtTA) which activates transcription of the gene of interest (Figure 1.3B) [28-31]. Regulatory proteins tTA and rtTA are encoded by pTet-Off and pTet-On regulator plasmids (Clontech), respectively, both of which contain a neomycin-resistance gene for selection.

The second critical component of the Tet systems is the response plasmid, which expresses a gene of interest under the control of the tetracycline-response element (TRE). The latest version of the pTRE-tight plasmid contains a modified TRE response element (TREmod) that consists of seven direct repeats containing *tetO* [31, 32]. In the pTRE-tight plasmid, TREmod resides upstream of an altered minimal cytomegalovirus (CMV) promoter ($P_{\mathrm{minCMV}\Delta}$) (Figure 1.3). In contrast to the complete CMV promoter, this minimal CMV promoter lacks the enhancer sequence allowing a reduction of the basal expression of the gene of interest. A Dox-dependent gene expression system with these two components, tTA and rtTA, is expected to display tightly controlled expression of the gene of interest. In the Tet-Off system, Dox represses expression (Figure 1.3A) while in the Tet-On system, Dox activates expression of the target gene (Figure 1.3B).

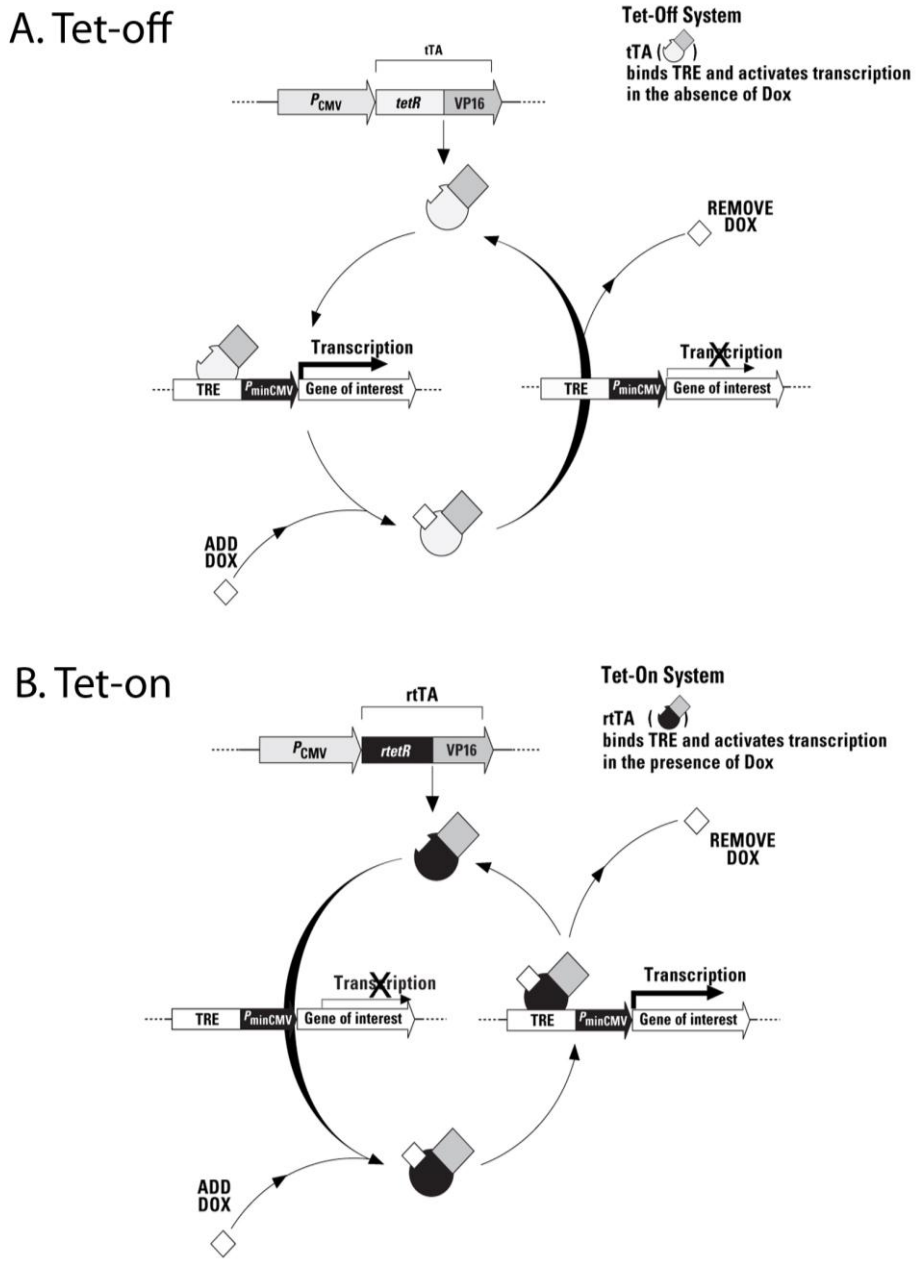

Figure 1.3: Schematic diagram of the gene regulation in the Tet-Off and Tet-On systems. The tetracycline response element (TRE) is located upstream of the minimal immediate early promoter of CMV ($P_{\text{minCMV}\Delta}$). **A. Tet-Off:** transcription of gene of interest remains active in the absence of Dox through tetracycline-conclled transactivator (tTA) binding to the TRE. Addition of Dox results in conformational changes in tTA and, therefore, disassociates tTA from the promoter to block gene transcription. **B. Tet-On:** transcriptional activator tetracycline-responsive transcriptional activator (rtTA) has a reverse response to Dox due to four amino acid changes compared to rTA. Transcription remains inactive without Dox. In the presence of Dox, rtTA undergoes conformational change and binds to TRE to activate gene transcription (Figure adapted from Clontech Laboratories user manual [33].

Both Tet systems allow stringent regulation of the target gene in a precise and dose-dependent manner. Some minor drawbacks still remain however, for instance, since these systems are derived from bacteria, they may illicit undesirable immunogenic responses when utilized in humans [20, 21]. In addition, Dox has been demonstrated to accumulate in the bone and teeth [34], which may delay the silencing of the Tet-On and activation of the Tet-Off systems [17, 21]. Conversely, a major drawback of the Tet-Off system is that patients would face long-term exposure to Dox for inactivation of the system which may lead to drug resistance and insensitivity. The re-induction of this system relies on the complete clearance of the drug and, therefore, the efficiency and effectiveness of this system could also be a problem. On the other hand, the Tet-On system requires a reasonably large dose of Dox to achieve activation of the target gene expression. In addition, the residual affinity of rtTA to *tetO* in this system may have an elevated leaky background activity [29, 30, 35]. This also poses a problem in stringent regulation especially in humans. To alleviate this issue, a mutant rtTA2^{S}-M2s has been developed which activates the Tet-On system at a 10-fold lower Dox concentration than that of wild type rtTA. The mutant rtTA2^{S}-M2s has been shown to offer greater stability and zero background expression in the absence of Dox [30, 35].

1.1.3 Applications of lentiviral systems in primary and stem cells

Lentiviral systems are widely used in gene therapies and as a research tool, especially in primary cells and stem cells mostly using a constitutive gene expression system. One of the most common uses of lentiviral vectors is to silence genes using RNA interference (RNAi) [36, 37]. Lentiviral vectors can establish a stable genetic modification of human embryonic stem (hES) cells [38], retain sustained transgene expression in hES cells [39], and induce the generation of pluripotent stem cells [40]. It has been reported that a lentiviral vector governs the stable transduction of cells to induce the reversible immortalization of human primary cells regardless of their cycling status [41]. Furthermore, inducible Tet-On lentiviral systems have been demonstrated to be able to avoid down-regulation of the target gene in *ex vivo* gene transfer to the central nervous system [42]. An adverse effect also exists however, in the *in vitro* application of lentiviral vectors [43]. Therefore, development of a novel Tet-On lentiviral system with an instant induction feature is critically required to overcome several drawbacks that the

current lentiviral gene delivery system has been suffering in primary and stem cell applications.

1.2 Molecular ageing in association with Protein phosphatase type 2C isoform beta (PP2Cβ)

Decline in the functional abilities of organisms, organs, tissues and cells occur during the ageing phase of their life span. Cellular senescence is a terminally differentiated cellular state, characterised by exhaustion of proliferative potential and permanent cell cycle arrest in human diploid primary cells. This process is also known as replicative senescence or the Hayflick phenomenon [44]. Since ageing accompanies diverse and multiple levels of changes in gene expression, investigating the mechanisms controlling age-dependent gene expression is likely to enhance understanding of the ageing phenomena at the molecular level. Replicative senescence is known to be induced by multiple signaling pathways and their resultant gene expression, which are governed by senescence-associated transcriptional regulators. This current study aims to provide a tool to study the role of protein phosphatase PP2Cβ in relation to molecular ageing.

1.2.1 Protein phosphatase type 2C isoform beta (PP2Cβ)

In cellular signal transduction, protein phosphorylation is one of the most important mechanisms mediated by a balance between protein kinases and cognate protein phosphatases [45]. Based on their structural and functional diversity, protein phosphatases can be classified into three major families: (i) protein tyrosine phosphatases (PTPs) that dephosphorylate phosphotyrosine residues; (ii) phospho-protein phosphatases (PPPs) containing a highly conserved catalytic domain and are further classified by distinct amino acid sequences, that dephosphorylate serine/threonine residues and are further divided into three distinct subtypes of phosphatases including type PP1, PP2A and PP2B in eukaryotes, and finally (iii) protein phosphatase magnesium-dependent (PPMs) enzymes that are characterised by their requirement of Mg^{2+} or Mn^{2+} ions for activity. PPMs mainly consist of pyruvate dehydrogenase phosphatase and PP2C, and dephosphorylate phosphoserine and phosphothreonine residues like PPPs [46-50].

PP2C belongs to the second family, PPPs, in eukaryotes, but shares little homology with other phosphatases PP1, PP2A and PP2B [51]. These three phosphatases share ~40% amino acid identity in their primary structure. In addition, they are sensitive to phosphatase inhibitors and are associated with their cognate regulatory subunits by forming heteroligomeric complexes [1, 52]. Unlike these three phosphatases, monomeric PP2C is a free catalytic protein that requires metal ions for activity. So far, no specific inhibitor of PP2C has been identified [1, 46, 48, 50]. In contrast, unsaturated fatty acids with special structural features like oleic acid are known to activate PP2C activity [48]. PP2C is able to dephosphorylate a broad spectrum of substrates involved in diverse cellular events and signaling pathways. There are at least 16 different PP2C genes encoding more than 20 distinct PP2C isozymes [45, 47, 53, 54]. Among those, the paralogs α and β were the first identified and have been studied in the greatest detail.

PP2Cβ has six distinct isoforms, β-1,-2,-3,-4,-5 and -X, which are produced from alternative splicing of a single pre-mRNA [46, 47, 55]. The amino acid sequences of these isoforms differ only at the C-terminal ends, suggesting that substrate specificity is determined by the C-terminus [47, 49, 56]. PP2Cβ is mostly localised in the cytoplasm however, isoforms β-1 and β-2 are also known to be present in the nucleus [45]. Expression in mice revealed that isoforms β-1, β-2 and β-X are expressed ubiquitously, and β-3,-4, and -5 are exclusively expressed in intestine, adult testis and liver cells [45-47].

The primary sequence of PP2Cβ shares 77% identity and 87% similarity to its paralog PP2Cα in mammalian cells. These proteins, PP2Cα and β share similar roles, for instance, they are involved in suppressing the stress activated protein kinase pathway. Their functions however, appear to be dependent on tissue type and stage of cell development [46, 49].

1.2.2 Known functions of PP2Cβ

Members of the PP2C family are known to lack regulatory subunits due to their monomeric structure [1, 46, 48, 50]. In living systems, the physiological concentration of divalent cations, such as Mg^{2+} and Mn^{2+}, which are required for PP2C function, remain constant and are difficult to manipulate hence the cation-dependent functionality

of PP2C proteins has been challenging to assess [1, 46, 47, 50]. PP2C phosphatases are known to play a role in cell proliferation, differentiation, and apoptosis and stress signaling pathways [1, 48, 52, 55, 57]. Overexpression of PP2Cβ in HEK 293T cells leads to cell-growth arrest or cell death [49]. Early reports suggested that the PP2Cβ mRNA level is increased during the first wave of spermatogenesis in pachytene spermatocytes and more highly differentiated germ cells, implying differentiation dependent regulation of PP2Cβ [58]. Later on, the same group confirmed that tissue-specific expression of PP2Cβ uses alternative promoters within the PP2Cβ gene to direct its expression [46]. Findings of later knock down studies, suggest that PP2Cβ may play a role in gametogenesis, fertilization and the early stages of embryonic development [47]. Although PP2Cβ is also believed to have critical roles in a number of pathways, very little is known about the molecular mechanisms behind PP2Cβ regulation.

1.2.2.1 Regulation of stress signaling pathways (SAPKs)

PP2Cβ is known to be involved in the regulation of the stress-activated protein kinases (SAPKs) pathways. SAPKs are a subfamily of the mitogen-activated protein kinase (MAPK) superfamily which is highly conserved from yeast to humans and respond to SAPKs stimuli including extracellular stress and inflammatory cytokines. There are two prominent classes of SAPKs in mammalian cells, C-Jun amino-terminal kinases (JNKs) and p38 kinases, which are activated by extracellular stresses through phosphorylation of conserved tyrosine and threonine residues in the catalytic domain by protein kinases belonging to the family of MAPK kinases (MKK) (Figure 1.4) [51, 54, 55]. JNK is predominantly phosphorylated by the MKK4 and MKK7 protein kinases while p38 is specifically phosphorylated by the protein kinases MKK3 and MKK6 [51, 55]. Interestingly, MKK4 can also phosphorylate p38. The MKKs are further activated by phosphorylation by MKK kinases (MKKK) which can directly sense extracellular stress signals. There are numerous MKK kinases (MKKK) such as apoptosis signal-regulating kinase 1 (ASK1), MAPK kinase kinase (MEKK) group (MEKK1, 2, 3, 4), MAP three kinase 1 (MTK1), mixed lineage kinase (MLK) family, transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) [45, 51, 55].

Upon a stress signal, MKK kinases (MKKK) activate MKKs which subsequently activate JNK or p38, followed by activation of transcriptional factors c-Jun or ATF2, thus, gene expression is changed to allow survival under stress conditions [45, 51, 54, 55]. PP2Cβ1 expression has been shown to suppress anisomycin- and NaCl- enhanced phosphorylation of p38, as well as the phosphorylation of MKK3b and MKK6b [55]. Overexpression of PP2Cβ1 reduces the basal and stress-enhanced phosphorylation of MKK4 and MKK7 in the JNK pathway [47, 55, 59, 60], suggesting that PP2Cβ is a negative regulator in SAPK pathways.

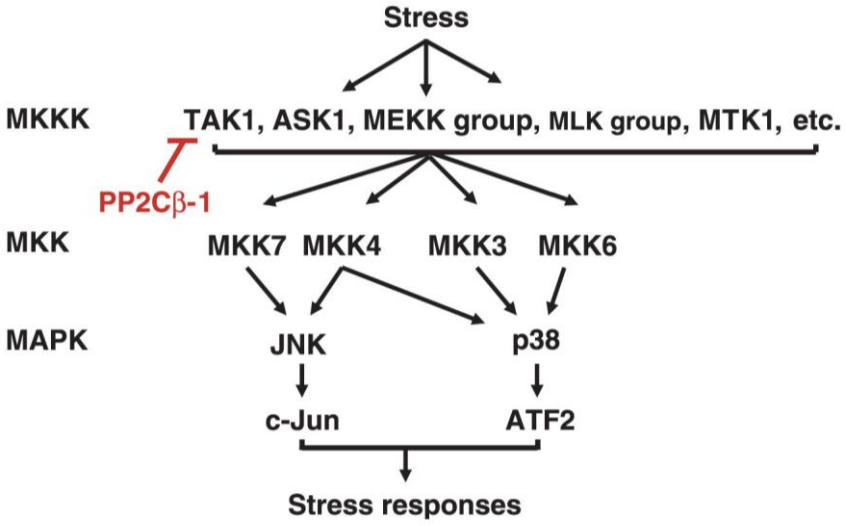

Figure 1.4: Regulation of stress-activated protein kinase (SAPK) signal pathways by PP2Cβ1. The protein kinase cascade of SAPK signaling pathways is shown, PP2Cβ1 interferes with MKKKs to regulate SAPK pathway. ASK1, apoptosis signal-regulating kinase1; ATF2, activating transcription factor; JUK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase kinase; MKK, motigen-activated protein kinase kinase; MKKK, mitogen-activated protein kinase kinase kinase; MLK, mixed lineage kinase; MTK1, MAP three kinase 1; PP2Cβ, protein serine/threonine phosphatase 2C isoform beta; TAK1, transforming growth factor β (TGFβ)-activated kinase 1 (Figure is modified from Tamura *et al.*, 2006 [54], permission for publishing obtained through Wiley).

Transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) was originally identified as a MKKK in the TGFβ signaling pathway. Previously, TAK1 was found to activate JNK and p38 pathways by phosphorylating MKK. More recent studies demonstrated that a phosphatase-negative mutant PP2Cβ1 acts as a dominant negative mutant to inhibit dephosphorylation of TAK1 by wild type PP2Cβ1, suggesting that the

expression of wild type intact PP2Cβ1 dephosphorylates and inactivates the TAK1 signal, preventing an efficient stress response [44, 47, 51]. These studies strongly support the concept that PP2Cβ functions as a negative regulator in SAPKs pathways.

1.2.2.2 Implication of PP2Cβ in regulation of cell division

In eukaryotes, cell cycle progression relies on the periodic activation and inactivation of cyclin-dependent protein kinases (CDKs) [52]. Activation of CDK is controlled by extracellular and intracellular signals, such as associated regulatory proteins (cyclins, inhibitors, and assembly factors), reversible protein phosphorylation, transcriptional regulation, selective proteolysis, and subcellular localization [61]. Full activation of CDK for normal cell cycle progression requires the recruitment of a cyclin, followed by the removal of inhibitory phosphate groups by Wee1-like protein kinases, and addition of activating phosphates in a region of the CDK called the "T-loop" by CDK-activating kinases (CAKs) [52, 62]. PP2Cβ2 and recombinant PP2Cβ2 have been shown to remove T-loop phosphates on CDK2 and CDK6 [45, 52]. Furthermore, it has been demonstrated that PP2C-like enzymes predominantly dephosphorylate human CDK2 in HeLa cell extracts suggesting that PP2Cβ is likely to be an important inhibitor in cell cycle progression [62].

1.2.2.3 Involvement in the regulation of apoptosis

Phosphorylation and dephosphorylation of proteins are essential to ensure proper cellular development and function, and has important implications in controlling cell apoptosis. For instance, phosphorylation of Bcl-2-associated death promoter (BAD) by 14-3-3 proteins keeps BAD in the cytosol and abolishes BAD-mediated pro-apoptotic responses. In contrast, dephosphorylation of BAD by PP2Cα and PP2Cβ induces cell death by heterodimerization with the anti-apoptotic oncogenes Bcl-2 and Bcl-X_L at the mitochondrial membrane [53, 63]. BAD belongs to the pro-apoptotic protein family of Bcl-2 gene and when de-phosphorylated interacts with anti-apoptotic molecules Bcl-2 and Bcl-X_L to promote apoptosis [53, 63, 64]. PP2Cβ dephosphorylates serine 155 in BAD which is critical for its interaction with Bcl-X_L in the mitochondrial matrix, suggesting that PP2Cβ has an important role in the regulation of mitochondrial apoptosis [63, 64]. It has been demonstrated that unsaturated fatty acids with special

structural features, such as oleic acid, activate PP2Cβ-dependent apoptosis in neuronal and endothelial cells [48, 53].

1.2.2.4 Involvement of PP2Cβ in molecular ageing through p53 pathway and NFκB signaling pathway

The pivotal tumour suppressor p53 is mainly regulated by its negative regulator Mdm2 through ubiquitination and subsequent proteasomal degradation [57, 65, 66]. In response to DNA damage, the interaction between p53 and Mdm2 is disrupted and the level of p53 is dramatically increased. Overexpression of PP2Cα, a close paralog to PP2Cβ, has been shown to enhance the stability of p53 resulting in higher expression of the p53 downstream target gene, p21, which is a key player in G2/M cell cycle arrest and apoptosis [57, 67]. PP2Cβ has also been proposed to increase the stability and activity of p53 by down-regulating Mdm2 [45, 57, 66], however, the mechanism of this is unclear. Recently, Park *et al.* have demonstrated that PP2Cβ associated with the brain cancer-amplified gene GAS41, which is a negative regulator of p53 tumour suppressor [65, 66]. The GAS41-PP2Cβ complex was shown to be required for dephosphorylation of p53 at serine 366 [66]. These studies suggest that PP2Cβ appears to be involved in both positive and negative regulation of the p53 pathway.

Nuclear factor kappa B (NFκB) belongs to a family of dimeric transcription factors containing a Rel homology domain (RHD) which are involved in numerous cellular pathways from cellular stress response to apoptosis and immunity [68-71]. NFκB is activated by a variety of stimuli including cytokines, toll-like receptors (TLRs), antigen receptors, G protein-coupled receptors (GPCRs), and growth factors [26, 72, 73]. In the absence of stimuli, NFκB is sequestered in the cytoplasm in an inactive form by its inhibitory binding partner, IκB. Upon stimulation, IκB is phosphorylated, ubiquitinated and eventually degraded by the proteasome, freeing NFκB and allowing it to translocate into the nucleus to activate its target genes [1, 70]. Cytokines such as tumor necrosis factor (TNFα), interleukin-1 (IL-1β) and transforming growth factors (TGFα) activate the phosphorylation of IκB by IκB kinases (IKKs) [1, 70]. The IKKs form complexes consisting of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IK γ /NEMO [70]. PP2Cβ is known to associate with the IKK complex upon cytokine stimulation. It has also been shown that PP2Cβ association with IKKβ, decreases IKKβ

kinase activity therefore terminating IKKβ-induced NFκB activation pathway [1]. This suggests that PP2Cβ down regulates cytokine-induced NFκB activation by altering IKK activity. A recent study confirmed that overexpression of PP2Cβ resulted in dephosphorylation of IKKβ while knocking down PP2Cβ expression promotes cytokine-induced NFκB activation by enhancing IKKβ phosphorylation [70]. In context with the multi-function of NFκB in cell survival, apoptosis and cellular stress signaling, PP2Cβ seems to play a role as an inhibitor of cellular progression and cellular stress signaling pathways.

NFκB is also known as a master regulator of gene expression programs associated with mammalian ageing [74, 75]. It is an ultimate effector that senses multiple age-associated signals from oxidative stress, DNA damage, cell survival and inflammation [71]. Constitutive activation of NFκB induces ageing phenotypes among various cell lines and animal models in which NFκB-dependent gene expression contributes deleterious effects of age-associated diseases such as induced insulin resistance and neurotoxicity in Alzheimer's disease [74, 76-78]. Blocking NFκB activity in aged cells has been shown to reverse many of these phenotypes by inhibiting age-associated gene expression [74]. PP2Cβ is known to play a role as a negative regulator of IKKβ terminating IKKβ-induced NFκB activation [1, 70]. PP2Cβ is a common regulator of NFκB and the p53 pathways which are likely to exhibit “cross-talk” with each other to coordinate precise control of gene expression in aged cells. Given a common negative regulatory role of PP2Cβ on p53 and NFκB transcription factors, it is conceivable that PP2Cβ would be significantly down-regulated in ageing process.

1.3 Background to this project

The original design of the tetracycline regulatory system consists of two plasmids. One plasmid expresses *rtTA* constitutively, and the other contains the gene of interest. This binary system has been combined into an autoregulatory plasmid which contains rtTA and the transgene [28, 31, 32]. This current study employed a bi-directional pTRE-Tight-BI lentiviral plasmid, coupled with the Tet-On system (Figure 1.5). This promoter consists of two mini CMV promoters flanking the TREmod element, in which rtTA and PP2Cβ, are under the control of their respective mini CMV promoters (Figure 1.5 a). In theory, almost no background levels of rtTA can be detected in this system without Dox

induction. To activate the mini CMV promoters, Dox is added to the system which forms a complex with rtTA (Figure 1.5 b). This Dox-rtTA complex binds to the TRE element activating the mini CMV promoters and the expression of the downstream genes, *rtTA* and *PP2Cβ* (Figure 1.5 c). As more rtTA is produced in the presence of Dox, more Dox-rtTA complexes form which bind the TRE element, further inducing the expression of PP2Cβ. To switch off this system, Dox is removed to silence both mini CMV promoters and terminate the expression of PP2Cβ (Figure 1.5 d).

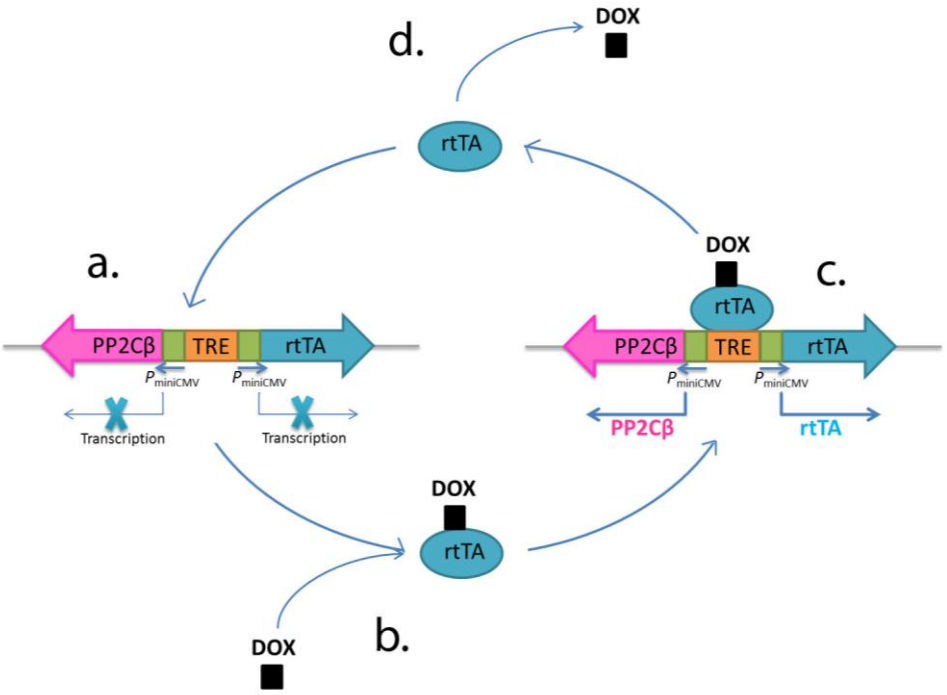

Figure 1.5: Activation of bi-directional lentiviral vector of PP2Cβ. a. Tetracycline response element (TRE) flanked with two mini CMV promoter, reverse tetracycline response transcriptional activator (rtTA) and the gene of interest PP2Cβ reside downstream of each mini CMV promoter. **b.** Addition of Dox to the system allows its binding to rtTA in a basal expression level to form an activator complex, Dox-rtTA. **c.** Binding of Dox-rtTA complex to TRE activates both mini CMV promoters and, therefore enhances the transcription of its downstream gene, *PP2Cβ* and *rtTA*, respectively. **d.** Removal of Dox from the system leads to disassociation of Dox-rtTA complex, thus, blocks the transcription activation.

This positive feedback regulatory promoter in the lentiviral system is thought to be superior to a normal constitutive promoter in a number of aspects, including higher viral titer production, undetectable levels of rtTA and the transgene in the Off state, improved induction kinetics, and repeatable induction of the target gene expression with multiple

cycles of Dox exposure [28, 32]. Most importantly, the induction of the transgene can be achieved within a single cassette using a bi-directional autoregulatory vector. Compared to the binary system in general Tet-On systems, this autoregulatory system has been shown to significantly reduce the process of establishing homogeneously transduced populations to prevent unwanted cytotoxicity and immunogenicity and thus preventing its utilization *in vivo* [31, 32]. An early study reported that the autoregulatory Tet-Off system could produce a possible adverse effect at cellular levels due to higher VP16 levels. After 48 h of rTA induction, cells have been observed to increase in size with a significant (over 50%) reduction in growth rate [31, 32]. Therefore, the expression of transcriptional regulators should be tightly regulated as well to minimize cellular toxicity.

1.4 Project outline

This study was undertaken to investigate the involvement of PP2Cβ in NFκB pathway-mediated cellular ageing by utilizing a Tet-on induced lentiviral system. PP2Cβ is implicated in a number of cell processes including, cell proliferation, differentiation, senescence and apoptosis. Importantly, recent studies revealed a chronological down-regulation of PP2Cβ during cellular senescence of human diploid fibroblast, suggesting that PP2Cβ plays a key role in cellular ageing process (unpublished result, Dr. Jeong Park).

This research aims to construct and verify the Dox-inducible lentiviral vectors for the stable expression of PP2Cβ [pLenti-Bi-TRE-Tet-On (V), pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT) and pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)] in immortalized cancer cells. These viral vectors will be used to determine the role of PP2Cβ in cellular senescence and regulation of NFκB activity in primary human diploid cells in the future.

1.4.1 Hypothesis

A basal expression of rtTA in the bi-directional TRE vectors allows instantly inducible expression of PP2Cβ without establishing a Tet-On cell line.

1.4.2 Research aim

The first aim of this study was to construct viral vectors for tetracycline inducible PP2Cβ (WT, MUT).

Objectives:

- Clone a T7-tag reverse tetracycline responsive transactivator (rtTA) into an empty lentiviral vector (V) or lentiviral vectors expressing PP2Cβ (WT) and PP2Cβ (MUT), respectively.
- Confirm instant inducibility of lentiviral vectors in HEK 293T cells by transient transfection upon Dox treatment.

The second aim was to examine PP2Cβ expression levels in lentiviral infected HEK 293T cells.

Objectives:

- Produce lentiviral stocks for vector only (V), PP2Cβ (WT) and PP2Cβ (MUT) in co-packaging cells.
- Examine the protein level of PP2Cβ in infected HEK 293T cells in the presence and absence of Dox.
- Compare PP2Cβ expression levels of WT and MUT using western blot and real-time quantitative PCR analyses.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General

Materials	Manufacturer/ Supplier
Agarose	Bioline, London, UK
Absolute ethanol, Boric acid, Chloroform, Methanol, Isopropanol	Merck, Darmstadt, Germany
Bovine serum albumin (BSA)	New England Biolabs, Ipswich, MA
Cellstar® centrifuge tubes (15 mL, 50 mL), Plastic pipettes (5, 10 and 25 mL)	Greiner Bio-One, Frickenhansen, Germany
E.Z.N.A.® Gel Extraction Kit	Omega bio-tek, Georgia, USA
Microcentrifuge tubes (1.7 mL, 0.6 mL), PCR tubes (0.2 mL), Pipette tips	Axygen,California, USA
B-mercaptoethanol, Bromophenol blue, Ethidium bromide Ethylenediaminetetraacetic acid (EDTA), DirectLoad™ Wide Range DNA Marker, Glycerol, Glycine, Magnesium chloride anhydrous, Polyethylen glycol (PEG), Potassium Chloride (KCl), Potassium phosphate monobasic, ACS reagent (KH_2PO_4), Sodium chloride (NaCl), Sodium fluoride (NaF), Sodium phosphate diabasic (Na_2HPO_4), Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma-Aldrich, Missouri, USA
Tris base	Carl Roth, Karlsruhe, Germany
Nanodrop®-spectrophotometer ND-1000	Thermo Fisher scientific, Massachusetts, USA
Gel Doc™ system	BioRad Laboratories, California, USA

2.1.2 Cloning

Materials	Manufacturer/ Supplier
Ampicillin trihydrate, Lysozyme, T4-DNA ligase and buffer	Sigma-Aldrich, Missouri, USA
Bovine serum albumin (BSA), Calf intestinal alkaline phosphatase (CIP) *ClaI*, *EcoRI*, *XmaI*	New England Biolabs, Ipswich, MA

LB Broth base, PureLink™ Hipure Plasmid Kit (Miniprep and Midiprep)	Invitrogen, California, USA
Petri dish	Thermo Fisher Scientific, Massachusetts, USA
RNase A, *XhoI*	Roche, Auckland, NZ

2.1.3 Cell culture and lentiviral preparation

Materials	**Manufacturer/ Supplier**
BD Plastipak™ 50 mL syringe	BD Biosciences, Temse, Belgium
Cellstar® cell culture dishes (60 mm, 100 mm), Cellstar® 24 well plate, Plastic pipettes (5, 10 and 25 mL)	Greiner Bio-One, Frickenhansen, Germany
Costar® 6 well cell culture cluster	Corning Incorporated, NY, USA
Caffeine, Crystal violet dye, Doxycycline hyclate, Puromycin, Polybrene	Sigma-Aldrich, Missouri, USA
Dulbecco's Modified Eagel Medium (DMEM), OPTI-MEM, Penicillin/streptomycin (Pen/Strep), Trypsin/EDTA	Invitrogen, CA, USA
Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS)	SAFC Biosciences, Hampshire, UK
FuGENE HD transfection reagent, X-tremeGENE HP transfection reagent	Roche, Auckland, NZ
Microtube 2 mL (Cryovials)	SARSTEDT, Nümbrecht, Germany
Minisart Syringe filter, hydrophilic, 0.45 µm	Sartorius stedim Biotech, Gottingen, Germany
Plasmocin	InvivoGen, CA, USA

2.1.4 RNA extraction

Materials	**Manufacturer/ Supplier**
Chloroform	Merck, Darmstadt, Germany
DNase, RNase free water	Roche, Auckland, NZ
Trizol® LS reagent	Invitrogen, California, USA

2.1.5 DNA extraction

Materials	Manufacturer/ Supplier
Phusion DNA polymerase and Phusion HF reaction buffer	Thermo Scientific, Waltham, USA
dNTP	Takara Bio, Shiga, Japan
Veriti™ 96 well Thermal Cycler	Applied Biosystems

2.1.6 Protein extraction and western blotting

Materials	Manufacturer/ Supplier
40% Acrylamide/bis-acrylamide solution 19:1, Bradford reagent, Mini PROTEAN® 3 gel casting system, Minigel apparatus, Mini Trans-Blot® filter paper	BioRad Laboratories, California, USA
Ammonium persulfate (APS), Dithiothreitol (DTT), N,N,N',N'-Tetramethylethylenediamine (TEMED), NP-40, Phenylmethanesulfonyl fluoride (PMSF), Sodium azide, Sodium dodecyl sulphate (SDS), Triton X-100, Tween-20	Sigma-Aldrich, Missouri, USA
Biomas general blue film, Biomas masximum sensitivity film, Biomas MS intensifying screen cassette 8" × 10"	Kodak, NY, USA
Microplate reader	BioTek, Vermont,USA
Goat Anti-Mouse IgG-HRP	Invitrogen, California, USA
PageRuler™ prestained protein ladder	Fermentas, Burlington, Canada
Pierce® ECL Western Blotting Substrate, SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo Scientific, Waltham, USA
Polyvinylidene fluoride (PVDF) membrane	GE Healthcare, Wisconsin, USA

2.1.7 Reverse transcriptase quantitative real-time PCR

Materials	Manufacturer/ Supplier
LightCycler® 480 SYBR Green I Master, LightCycler® 480 instrument	Roche, Auckland, NZ
SuperScript® Platinum® *Taq* Mix	Ivitrogen, California, USA
Frame Star 480 Q-PCR plate (96-well)	4titude®, Ockley, UK

2.2 Methods

2.2.1 Cloning

The cDNA of reverse tet-responsive transcriptional activator (rtTA) was first amplified from the pTet-On plasmid (Clontech) using conventional PCR amplification (Section 2.2.5.1). In the Tet-on system [28-31], rtTA binds to the tet-responsive element (TRE) and activates the transcription in the presence of doxycycline. The *rtTA* PCR product was sub-cloned into three bi-directional TRE-inducible lentiviral vectors (pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT; previously constructed by Dr. Jeong Park) to control the expression of PP2Cβ in response to Dox.

2.2.1.1 Restriction endonuclease digestion

Three bi-directional plasmids and the *rtTA* PCR product were digested with two restriction endonucleases. Most digestions were carried out overnight at 37ºC. Reaction mixtures consisted of 2 µg of DNA, 0.5-1 µL of each restriction endonuclease (10 U/µL), 2 µL of 10 × bovine serum albumin (BSA, 1mg/mL) and 2 µL 10 × recommended buffer, in a total volume of 20 µL. Reaction mixtures were analysed by agarose gel electrophoresis (Section 2.2.5.2) and the desired DNA fragments were excised from the gel, followed by gel purification and PCR clean-up for subsequent cloning.

2.2.1.2 Calf intestinal alkaline phosphatase (CIP) treatment

In order to reduce self-ligation of the vector, plasmids were treated by incubation with 0.5 µL CIP (10 U/µL) at 37ºC for 1 h after restriction endonuclease digestion.

2.2.1.3 Ligation

Ligation was performed using T4 DNA ligase (Invitrogen). A vector:insert molar ratio of 1:3 was used to set up each ligation. Reactions were performed in a total volume of 10 µL containing 0.5 µL ligase (400 U/µL) and 1 µL of 10 × ligase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM ATP, pH 7.5/25ºC). The ligation mixture was incubated at 16ºC or room temperature overnight.

2.2.1.4 Transformation

Transformation was carried out using *E.coli* DH5α competent cells (in 80 mM $CaCl_2$, 10% glycerol). Two µL of ligation reaction mixture was added into 50 µL of competent cells and incubated for 30 minutes on ice, then heat-shocked at 42ºC for 1 minute followed by 2-3 minutes incubation on ice. Two hundred µL of sterile LB broth was added to the transformation mixture, and incubated at 37ºC for 1 h with constant shaking. The transformation mixture was transferred onto LB agar plate supplemented with ampicillin (50 µg/mL). Plates was incubated at 37ºC overnight. These procedures were carried out using the aseptic technique.

2.2.1.5 Inoculation

Single colonies were picked into LB supplemented with ampicillin (50 µg/mL), 3 mL of LB for small scale or 200 mL of LB for medium scale plasmid extraction, and incubated at 37ºC overnight with constant shaking for plasmid preparation.

2.2.1.6 Manual small scale plasmid preparation

Manual small scale plasmid preparation [79] is an economic and efficient way to check successful sub-cloning immediately after inoculation. In general, 1.5 mL of *E.coli* culture was pelleted by centrifugation at 12,000 rpm for 1 minute. The cell pellet was resuspended in 350 µL of STET buffer (0.1M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8) and 25 µL of freshly prepared lysozyme (10 mg/mL in sterile water), then boiled for 40 seconds followed by immediate centrifugation at 12,000 rpm for 10 minutes. The pellet was removed using a toothpick and discarded. Four hundred µL of isopropanol was added to the supernatant and incubated at -20ºC for 20-30 minutes to precipitate the DNA. Centrifugation at 12,000 rpm for 5 minutes was performed and the supernatant was removed. Another 500 µL of cold 95% ethanol was added to wash the pellet followed by centrifugation at 12,000 rpm for 1 minute, and the supernatant was removed. The pellet was air dried at room temperature and resuspended in 50 µL of TE (10 mM Tris, pH 8.0, 1 mM EDTA) containing 0.1 mg/mL of RNase A. Purified plasmids were then digested with restriction endonucleases and electrophoresed to identify recombinant plasmids.

2.2.1.7 Medium scale plasmid preparation

Once a *E.coli* colony was confirmed to have the plasmid of interest by manual plasmid preparation, LB supplemented with ampicillin (50 µg/mL) was inoculated with the appropriate clone overnight at 37ºC with constant shaking. Overnight cultures were centrifuged at 2,600 g at 4ºC for 20 minutes to pellet the plasmid DNA. Medium scale plasmid preparation was carried out using PureLink™ HiPure Plasmid Midiprep Kit according to manufacturer's instructions. Plasmid DNA was eluted with 800 µL of sterile TE.

2.2.1.8 Polyethylene glycol (PEG) purification

PEG purification was performed after medium scale plasmid preparation to increase DNA purity. NaCl (5M) and 300 µL of PEG solution (30% PEG 8000) were added to the plasmid DNA, and incubated on ice for 30 minutes followed by centrifugation at 16,000 × g at 4ºC for 15 minutes. The supernatant was discarded and the pellet was spun with additional 1 minute at 16,000 × g followed by removal of residual liquid. Five hundred µL of 70% ethanol was added to the pellet and centrifuged at 16,000 × g for 5 minute. The supernatant was aspirated in aseptic hood, the pellet was air dried for 10 minutes then resuspended in 200 µL sterile TE buffer. Concentration of purified plasmid DNA was determined using Nanodrop® ND-1000 spectrophotometer (Section 2.2.5.4).

2.2.2 Cell culture

The human embryonic kidney (HEK) 293T cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% v/v fetal calf serum (FCS) or fetal bovine serum (FBS), 0.5% v/v penicillin (5000 U/mL) and streptomycin (500 µg/mL) (Pen/Strep). Virally transduced HEK 293T cells were cultured in supplemented DMEM containing 1 µg/mL of puromycin. Addition of plasmocin (5-10 µg/mL) and modification of the medium contents were also applied to some procedures. All cells were cultured at 37ºC with 5% CO_2 in a humidified atmosphere. All tissue culture procedures were carried out in an ESCO Class II Biohazard safety cabinet.

2.2.2.1 Starting cell line

Frozen stocks (1 mL) were thawed and transferred into 5 mL of supplemented DMEM pre-warmed to 37ºC. The mixture was centrifuged at 200 × g for 5 minutes to pellet the cells. The cell pellet was gently resuspended in 2 mL DMEM and seeded into a 6 cm plate in a total volume of 4 mL.

2.2.2.2 Cells passaging

After growth medium was removed from the plate, the monolayer was washed once with 1×PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4, 10 mL/10 cm plate), and 0.5×Trypsin/EDTA (1 mL/10 cm plate) was applied to the monolayer, swirled and immediately removed. The plate was left to stand for 2-5 minutes at room temperature to allow sufficient trypsinisation. The side of the plate was tapped to ensure complete dislodgement. Cells were resuspended in supplemented DMEM (10 mL/10 cm plate) and an aliquot of 1 mL cell suspension were transferred to a fresh 10 cm plate or 6 cm plate containing ~9 mL or ~3 mL of growth medium, respectively.

2.2.2.3 Medium change

In cases where a cell culture had not reached ~60% confluency over 3 days of passage, medium was removed by aspiration and replaced with fresh growth medium.

2.2.2.4 Freezing cells

Cells at approximately 80% confluence after the first passage were used for freezing stocks. Cells were dislodged as described in Section 2.2.2.2, resuspended in 5 mL of growth medium and transferred into 15 mL centrifuge tube for 5 minutes centrifugation at 1000 rpm. The cell pellet was then resuspended in ~2 mL/3 mL freezing solution (10% DMSO, 20% FBS and 70% DMEM) for one 6 cm/10 cm plate, then dispensed 1 mL aliquots into two sterile cryovials. Cryovials were well wrapped with tissue paper to allow slow freezing in the -80 ºC freezer.

2.2.2.5 Transient transfection

X-tremeGENE HP DNA Transfection Reagent (Roche) was used to carry out transient transfection reactions. HEK 293T cells were seeded onto 6 cm plate and incubated until they researched ~60% confluency. In a sterile microcentrifuge tube, pre-warmed OPTI-MEM (200 µL/6 cm plate) was dispensed with 1 µg of DNA and 3 µL of transfection reagent, briefly tapped and incubated for 15 minutes at room temperature to allow formation of a transfection complex. Transfection mixture was then transferred onto designated 6 cm plate.

2.2.2.6 Viral transduction and puromycin selection

Lentivirus stocks were prepared as described in Section 2.2.3. HEK 293T cells were seeded onto 24 well plate (approximately 1×10^4 cells per well with 0.4-0.5 mL growth medium) and allowed to grow for 24 h. Growth medium was replaced with 400 µL of lentivirus transduction mixture (Table 2.1) and 0.1% v/v of polybrene (8mg/mL) was added to increase the efficiency of viral transduction. After 24 h of transduction, mixture was replaced with puromycin-containing medium to allow selection for successfully transduced cells. Once ~80% confluency was reached under puromycin selection, cells were ready to be harvested or treated for downstream experiments.

Table 2.1: Component of lentivirus transduction mixture for each vector.

Lentivirus	Virus Titer* (µL)	Supplemented DMEM (µL)	Polybrene (µL)
pLenti-Bi-TRE-Tet-on	100	300	0.4
pLenti- Bi-TRE-Tet-on-PP2Cβ WT	100	300	0.4
pLenti-Bi-TRE-Tet-on-PP2Cβ MUT	100	300	0.4

* Viral titer is expressed as a volume of a viral titer, eg. 100 µL of XX cfu/mL**.**

2.2.2.7 Dox induction of TRE-controlled constructs

Doxycycline induction was performed on > 80% confluent monolayers by adding 1 µM Dox (1 mg/mL) to the medium. In general, Dox treatment was performed 24 h post-

transfection, 24-72 h post-transduction or upon reaching ~ 80% confluency for stable cell lines derived from lentiviral infection. Dox was, unless otherwise stated, removed from the medium 24 h post-transfection.

2.2.3 Lentiviral preparation and optimisation

Lentiviral preparation and optimisation was carried out in an ESCO Class II Biohazard safety cabinet using sterile solutions and reagents. HEK 293T cells were used as a producer packaging cell line.

2.2.3.1 Packaging

Preparation: One day before co-transfection, a 10 cm plate of HEK 293T cells was prepared for each lentiviral vector using 10 mL of DMEM medium supplemented with 10% FBS per plate. Cells were seeded to a final density of 1.5×10^6 cells per plate (approximately 1:8 split), and incubated at 37ºC with 5% CO_2 in a humidified atmosphere.

Co-transfection: After 24 h incubation, cells at ~80% confluency were co-transfected with the desired plasmid DNA and packaging mix (provided by Dr. Jeong Park) using FuGENE® HD Transfection Reagent (Roche). In a sterile microfuge tube, pre-warmed OPTI-MEM (500 µL/10 cm plate) was dispensed with 5 µg of desired plasmid DNA, 3 µg of packaging plasmid mix and 24 µL of transfection reagent, briefly tapped and incubated for 15 minutes at room temperature to allow formation of transfection complex. Transfection mixture was then transferred onto designated 10 cm plate, and incubated for 17 h at 37ºC with 5% CO_2 in a humidified atmosphere.

Medium change: The initial medium was replaced with 10 mL viral harvest medium containing DMEM supplemented with 10% FBS, 1% Pen/Strep and 10 µg/mL of plasmocin (25 mg/mL). Caffeine was added to the medium to a final concentration of 1 mM to increase viral titer [80]. Viral harvest medium supplemented with caffeine was incubated for 24 h at 37ºC with 5% CO_2 in a humidified atmosphere for collection.

Collection: Viral harvest medium containing viral particles was collected into a 50 mL falcon tube every 24 h for three days. Each plate was refilled with 10 mL of fresh viral harvest medium after each collection for further harvest.

2.2.3.2 Concentration

The harvested viral soup was centrifuged at 3000 × g for 5 minutes, then its supernatant was removed and filtered through a 0.45 µm filter. Six mL of 50% PEG 6000 and 3 mL of 4 M NaCl (final 0.3 M) were added to the sterile viral solution. The mixture was thoroughly mixed and stored at 4ºC for 1.5 h followed by centrifugation at 7000 × g for 10 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended in 1.1 mL of DMEM, followed by incubation at 4ºC overnigh. This overnight stock was gently mixed by a pipette, and aliquots of 200 µL were placed into sterile cryovials for storage at -80ºC freezer. The residual stock was stored at 4ºC for titration.

2.2.3.3 Titration

Two 6-well plates of HEK 293T cells were prepared at a density of 1×10^5 cells per well. Ten-fold serial dilutions with 3 mL of growth medium and 8 µg/mL of polybrene according to Table 2.2 were prepared for three viral stocks. Cells were incubated at 37ºC with 5% CO_2 in a humidified atmosphere. After 24 h of incubation, initial growth medium was replaced with 2 mL virus containing medium in each well according to Table 2.2, and incubated for another 24 h. Lentivirus containing medium was replaced with 2 µg/mL puromycin selection medium to assess successful viral transduction. For selection, 2 µg/mL of puromycin selection medium was replaced every two days for the first week and reduced to 1 µg/mL of puromycin to replace every two days for another two weeks. After a total of three week growth, cells were stained with 1 mL 0.5% crystal violet solution in 20% ethanol and incubated for 30 minutes at room temperature. Each well was washed twice with distilled water. The number of blue-stained colonies was counted in each well. The functional viral titer was calculated according to the formula: (#colonies/2mL medium) × dilution factor = cfu/mL viral stock.

Table 2.2: Ten-fold serial dilutions of three viral stocks.

Loading Positions	10-fold serial dilutions		
Plate 1, well 1-3	Tet-on 1:1,000	WT 1:100	MUT 1:100
Plate 1, well 4-6	Tet-on 1:10,000	WT 1:1,000	MUT 1:1000
Plate 2, well 1-3	Tet-on 1:100,000	WT 1:10,000	MUT 1:10,000
Plate 2, well 4-6	Tet-on 1:1,100,000	WT 1:100,000	MUT 1:100,000

2.2.4 RNA

2.2.4.1 Phenol/chloroform extraction

Human HEK 293T cells were grown in 6 cm or 10 cm plates. Growth medium was aspirated from each plate, and 1 mL of PBS was added for harvest. Cells were harvested in PBS with a rubber scraper. Trizol® LS reagent (Invitrogen) was used to extract RNA from a liquid source. A 250 μL aliquot of cell suspension was transferred to a sterile microcentrifuge tube with 10 μg of glycogen, and 750 μL of Trizol® LS reagent was added to the tube, mixed and incubated at room temperature for 5 minutes. After incubation, 200 μL of chloroform (Merck) was added into the tube followed by vigorous vortex for 15 seconds and incubated at room temperature for another 5 minutes. The tube was centrifuged at 12,000 × g for 15 minutes at 4ºC. The colourless upper aqueous layer was transferred into a new RNase-free microcentrifuge tubes and an equal volume of isopropyl alcohol was added for subsequent purification.

2.2.4.2 RNA purification

The tube containing RNA was centrifuged at 12,000 × g for 10 minutes at 4ºC. Supernatant was removed and the RNA pellet was washed once with 1 mL of 75% ethanol for 5 minutes followed by centrifugation at 12,000 × g for 10 minutes at 4ºC. Pellet was air-dried for 30 minute and dissolved in 50 μL of RNase free water.

2.2.4.3 RNA quantification and quality assessment

The amount of RNA was quantified from the absorbance at 260 nm measured using the Nanodrop® ND-1000 spectrophotometer. RNA purity was assessed by the OD_{260}/OD_{280}

ratio and the OD_{260}/OD_{230} ratio generated by the Nanodrop® ND-1000 spectrophotometer. The RNA $OD_{260/280}$ ratio less than 2 indicates possible contamination by DNA, protein or phenol, because these molecules absorb electromagnetic radiation at a wavelength of around 280 nm. The $OD_{260/230}$ is a secondary measure of nucleic acid purity with uncontaminated RNA having an $OD_{260/230}$ greater than 1.8. A lower $OD_{260/230}$ ratio may indicate the presence of co-purified contaminants.

2.2.4.4 DNase treatment

Potential contamination by genomic DNA in the RNA sample was eliminated by DNase treatment. RNA samples were normalised to the lowest RNA concentration, and 45 µL of normalized RNA sample was transferred to 0.6 mL PCR tubes. One µL of DNase and 5 µL of 10 × DNase buffer were added, followed by incubation at 37ºC for 30 minutes and then at 75ºC for 5 minutes for DNase inactivation.

2.2.5 DNA

2.2.5.1 PCR

The cDNA of the *rtTA* insert was initially amplified from the pTet-On plasmid (Clontech) using conventional PCR. PCR amplification was performed using the Veriti™ 96 well Thermal Cycler (Applied Biosystems). A total of 50 µL of reaction cocktail contained 0.5 µL of Phusion DNA polymerase (2U/µL), 10 µL of 5 × Phusion HF reaction buffer (contains 7.5 mM $MgCl_2$), 5 µL of 10 × dNTP (2.5 mM) , 2.5 µL of each forward (10 µM) and reverse (10 µM) primer, 10 ng/µL of pTet-On plasmid DNA template and distilled water. A negative control containing no DNA template was included.

The standard PCR amplification cycling steps were as follows:

Enzyme activation	95°C for 1 minutes	
Denaturation	95°C for 15 seconds	35 × cycles
Annealing	54°C for 30 seconds	
Extension	72°C for 1 minutes	
Final Extension	72°C for 5 minutes	

2.2.5.2 Agarose gel electrophoresis

PCR and digestion products were analysed by horizontal electrophoresis using 0.8% to 2% w/v agarose gels in 0.5 × TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). Samples were loaded into designated wells with the addition of 1 µL of 6 × DNA loading dye (0.25% Bromophenol blue, 50% w/v glycerol). Electrophoresis was carried out at ~120 V for 1 h, and the gel was thereafter immersed in ethidium bromide (0.5 µg/mL) staining solution for 12 minutes. After destaining in water for 2 minutes, bands were visualised under ultraviolet light on the Gel Doc™ system (BioRad) and the sizes of the PCR products were compared to the DirectLoad™ Wide Range DNA Marker (Sigma).

2.2.5.3 Gel extraction and PCR purification

Desired bands were excised from agarose gel, and purified using E.Z.N.A.® Gel Extraction Kit (Omega) according to manufacturer's instructions. PCR and some digestion products were also purified using this kit. For vector size over 5000 bp, elution buffer were heated to 65°C prior to elution. A final volume of 30 µL of DNA was eluted.

2.2.5.4 DNA quantification

DNA was quantified by the absorbance at 260 nm measured with the Nanodrop® ND-1000 spectrophotometer. DNA purity was assessed by the OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios.

2.2.6 Protein analysis

2.2.6.1 Protein extraction

Growth medium was aspirated from each plate (usually 6 cm plate), and 1 mL of PBS was added for harvest. Cells were harvested with a rubber scraper. The dislodged cells were transferred to a sterile microcentrifuge tube, and centrifuged at 3000 rpm for 5 minutes at 4ºC. All traces of supernatant were aspirated after centrifugation. The pellet in each tube was resuspended in 100 µL of NP-40 lysis buffer (500 mM NaCl, 50 mM Tris pH 7.5, 0.2% NP-40, 10% glycerol) containing 1 mM of serine protease inhibitor

phenylmethanesulfonyl fluoride (PMSF, 100 mM), and incubated on ice for 10 minutes followed by a briefly vortex. Protein lysate was centrifuged at full speed for 5 minutes at 4ºC. The supernatant representing the protein extract was transferred into a new sterile microcentrifuge tube for storage at -20ºC.

2.2.6.2 Cell fractionation for protein manipulation

Cells were harvested, as described in Section 2.2.6.1, and centrifuged at 3000 rpm for 5 minutes at 4ºC to pellet the cell. Cell pellet was gently resuspended in 0.5 mL buffer A (10 mM HEPES, pH7.5, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF, and were immediately spun at 3000 rpm for 5 minutes at 4ºC. The swollen pellet was resuspended in 50 µL of buffer A. Another 50 µL of buffer A containing 1% NP-40 was added to the cell suspension, gently mixed by tapping and incubated on ice for 10 minutes. After incubation, the mixture was spun at 6,500 rpm for 10 minutes, and the supernatant was collected as a crude cytoplasmic fraction. The pellet was further submerged in 50 µL of F buffer (25 mM Tris, pH 7.5, 50 mM NaCl, 30mM $Na_4P_20_7$, 50 mM NaF, 10% glycerol, 0.5% Triton X-100), vortexed for 1 minute and incubated on ice for 30 minutes. Another 1 minute vortex was performed followed by centrifugation at 16,000 for 10 × minutes. The supernatant was collected as nuclear fraction.

2.2.6.3 Bradford protein quantification

A protein standard curve from 0-1 mg/mL was prepared from Bovine Serum Albumin (BSA) stock (10 mg/mL) using Bradford reagent in 96-well plates. Each well contained 10 µL of pre-determined BSA standard or target protein sample and 200 µL of 1:5 diluted protein assay dye reagent concentrate (BioRad). All BSA samples and target protein samples were prepared in triplicate and loaded onto 96-well flat-bottom microplates. Samples were incubated at room temperature for 5 minutes to allow colour development, and their absorbance was measured at 595 nm using a spectrophotometer microplate reader (BioTek). The Gene5^{TM} software was used for analysis and a standard curve was generated in Excel (Microsoft office 2010).

2.2.6.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Target protein samples were assessed using 8% or 10% SDS-PAGE gel electrophoresis. Mini PROTEAN® 3 gel casting system (BioRad, 1.5 mm-spaced) was filled with ~7.5 mL of 8% resolving gel, followed by ~2.5 mL of 4% stacking gel on top of the polymerised resolving gel with an appropriate comb. The components of resolving and stacking gel are listed in Table 2.3. Protein samples were prepared with 1 × SDS loading buffer (33 mM Tris pH6.8, 1.7% SDS, 5% glycerol, 0.05% w/v bromophenol blue, 0.7 M β-mercaptoethanol) and denatured for 3 minutes at 100ºC. Denatured samples and PageRuler™ prestained protein ladder (Thermo Scientific) were loaded into designated wells in a minigel apparatus (BioRad), and electrophoresed at a constant voltage of ~130 V for 1.5 h in protein running buffer (25 mM Tris, 200 mM glycine, 0.1% w/v SDS).

Table 2.3: Components of 8% and 10% SDS-PAGE gel.

Components	**Resolving Gel (10 mL)**		**Stacking Gel (4 mL)**
	8%	**10%**	**4%**
H_2O	5.5 mL	5.0	2.6 mL
4×Tris/SDS, pH 8.8	2.5 mL	2.5	-
4×Tris/SDS, pH 6.8	-	-	1 mL
40% Acrylamide	2 mL	2.5 mL	0.4 mL
10% APS	100 µL	100 µL	40 µL
TEMED	20 µL	20 µL	8 µL

2.2.6.5 Western blotting

SDS-PAGE gel was soaked in transfer buffer (2 M Tris, 192 mM glycine, 20 % v/v methanol) for at least 5 minutes and electro-transferred onto a positively charged PVDF membrane (pre-soaked in methanol) at a constant current of 150 mA for 2.5 h. The membrane was incubated in blocking buffer (5% w/v skim milk powder, 0.02% sodium azide, TBST: 10mM Tris pH7.6, 150 mM NaCl, 0.2% v/v Tween-20) for 1 h at room

temperature on an orbital shaker (Labnet). Membrane was briefly rinsed with TBST, and incubated with the primary antibodies overnight at 4ºC on a shaker. Primary antibodies of target proteins were diluted 1:500 in TBST containing 0.04% blocking solution and 0.001% sodium azide. β-actin was used as a loading control with 1:1000 dilution containing 0.004% blocking solution and 0.001% sodium azide. Membrane was washed 3 × 15 minutes in TBST and incubated with HRP-conjugated secondary antibody (1:10,000 dilution) for 30 minutes at room temperature on a shaker, followed by 4 × 10 minutes wash steps in TBST. Membrane was developed using Pierce® ECL substrate (Thermo Scientific) according to the manufacturer's instructions and immunoreactive bands were detected on medical X-ray films (Kodak) using an automated film processor according to the manufacturer's instructions.

2.2.6.6 Membrane stripping and reprobing

Membranes were left wet in TBST prior to stripping. For membrane stripping, TBST buffer was removed and the membrane was incubated with stripping buffer (0.2 M Glycine, pH2.5-3, 0.05% Tween 20) in a 75ºC water bath for 20 minutes. Membranes were washed 2-3 times for 10 minutes with TBST and blocked with blocking buffer as described in Section 2.2.6.5 for 30 minutes prior to reprobing.

2.2.7 Reverse transcriptase-coupled quantitative real-time PCR (RTqPCR)

Reverse transcriptase-coupled quantitative real-time PCR amplifications were performed on the Lightcycler® 480 real time PCR instrument (Roche) according to the manufacturer's instructions. The synthesis of first strand cDNA of *rtTA* and subsequent quantitative real-time PCR was conducted using one-step RT-qPCR analysis (Roche LightCycler® 480 SYBR Green Master I). Three oligonucleotide primer pairs were designed using the Roche LightCycler® Probe Design 2.0 software (Table 3.2) Oligonucleotide primers were received as dry powder and resuspended in distilled water to make a 100 μM stock and then diluted further to 10 μM working concentration for the assays. The specificity of the designed oligonucleotide primer pairs were tested by conventional PCR (Section 2.2.5.1) and visualized on agarose gel electrophoresis (Section 2.2.5.2).

2.2.7.1 Quantitative real time PCR

Quantitative real time PCR cocktails were made in a volume of 18 µL, containing 10 µL of 2 × LightCycler® 480 SYBR Green Master I, 0.2 µL of reverse transcriptase mix, 2 µL of 10 × forward and reverse primer mix (10 µM) and 5.8 µL of RNase free water. Two µL of RNA template was added to the SYBR Green master cocktail for PCR amplification. LightCycler® 480 SYBR Green Master I specific cycling conditions on the Lightcycler® 480 real time PCR system were performed according to the manufacturer's instructions (Table 2.4). First strand cDNA synthesis was performed prior to PCR amplification and forty cycles were used for amplification.

Table 2.4: PCR cycling conditions for the amplification of *rtTA*.

Mode	Cycles	Target (ºC)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (ºC/s)	Acquisiton (per ºC)
cDNA synthesis	1	50	None	0:10:00	4.4	-
Pre-incubation	1	95	None	0:05:00	4.4	-
Amplification	40	95	None	0:00:10	4.4	-
		60	Single	0:00:30	2.2	-
Melting Curve	1	65	None	0:00:01	4.4	-
		95	Continu-ous	-	0.11	5
Cooling	1	40	None	0:00:30	2.2	-

2.2.7.2 Relative quantification and statistical analysis

Three reverse transcription repeats were carried out for each sample, and each sample was tested in triplicate. A standard curve with crossing point (X axis) verses Log concentration (Y axis, Log 2000, Log 200, Log 20) was constructed for both *rtTA* and *GUSB* (Appendix VI). Relative Log concentration of each sample was calculated from their standard curves and the formula shown in Appendix VII, which were constructed using Ct values generated from RT-qPCR reactions. Averages of relative Log concentration were calculated for each sample from their triplicate. Relative expression of each sample was calculated according to the formula $Relative\ expression = 10^{Relative\ Log\ Conc.}$. A normalization ratio based on a reference gene, *GUSB* expression between

induced (Dox) and uninduced (No Dox) cells (Normalization ratio = Uninduced/Induced *GUSB* expression) was calculated, where the normalization ratio of uninduced cells was set to be 1. This normalization ratio for each sample was used to calculate relative target gene expression of *rtTA*. A relative fold difference was calculated from normalized expression levels of *rtTA*. Student's t test was used to determine whether gene expression in induced cells was statistically different to that in uninduced cells.

Chapter 3: Results

3.1 Construction of inducible lentiviral vectors

3.1.1 Introduction

An efficient gene delivery vector should include two components: (1) a delivery plasmid containing a gene of interest, and (2) a regulatory system that manipulates expression of the gene of interest in a drug inducible manner. Lentiviral vectors are superior to other retroviral vectors in their ability to deliver a gene of interest into non-dividing cells as well as dividing cells. The main aim of this study was to construct three inducible lentiviral plasmids [pLenti-Bi-TRE-Tet-On (V), pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT) and pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)] which place the gene of interest under the control of the tetracycline dependent system, Tet-On.

3.1.2 Cloning strategies

A two-step strategy was implemented to construct these plasmids. The cDNA of reverse tetracycline-responsive transcriptional activator (*rtTA*) was PCR amplified from pTet-On plasmid (Appendix II and Figure 3.1). In the Tet-On regulatory system, rtTA is sensitive to the presence of doxycycline and binds to tetracycline response element (TRE) to activate the transcription of adjacent genes [28-31]. Oligonucleotide primer sequences were designed and synthesized (Table 3.1), which incorporate the restriction endonuclease (RE) sites, *XmaI* and *ClaI*, located upstream and downstream of the cDNA of *rtTA* respectively, to enable insertion into bi-directional lentiviral vectors (Appendix III). A T7 tag was appended in the forward primer sequence to facilitate easy detection of rtTA protein expression. Once the *rtTA* fragment was amplified from the pTet-On plasmid, the next step was to sub-clone this T7-tag containing *rtTA* fragment individually into each bi-directional lentiviral vector [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] through *XmaI* and *ClaI* sites (Figure 3.1). PP2Cβ MUT construct differs from WT construct by a single base pair substitution at R179G resulting in mutation of arginine to glycine (Appendix V) [1]. Three pLenti-Bi plasmids [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] were linearized by double-digestion with *XmaI* and *ClaI* at their multiple cloning sites (MSC) (Left, Figure 3.1). The amplified *rtTA* fragment, containing *XmaI* and *ClaI* site,

was digested with these enzymes to allow insertion into pLenti-Bi plasmids (Right, Figure 3.1). Construction of V, WT and MUT was attempted by ligation of *rtTA* with the pLenti-Bi plasmid at *XmaI* and *ClaI* sites.

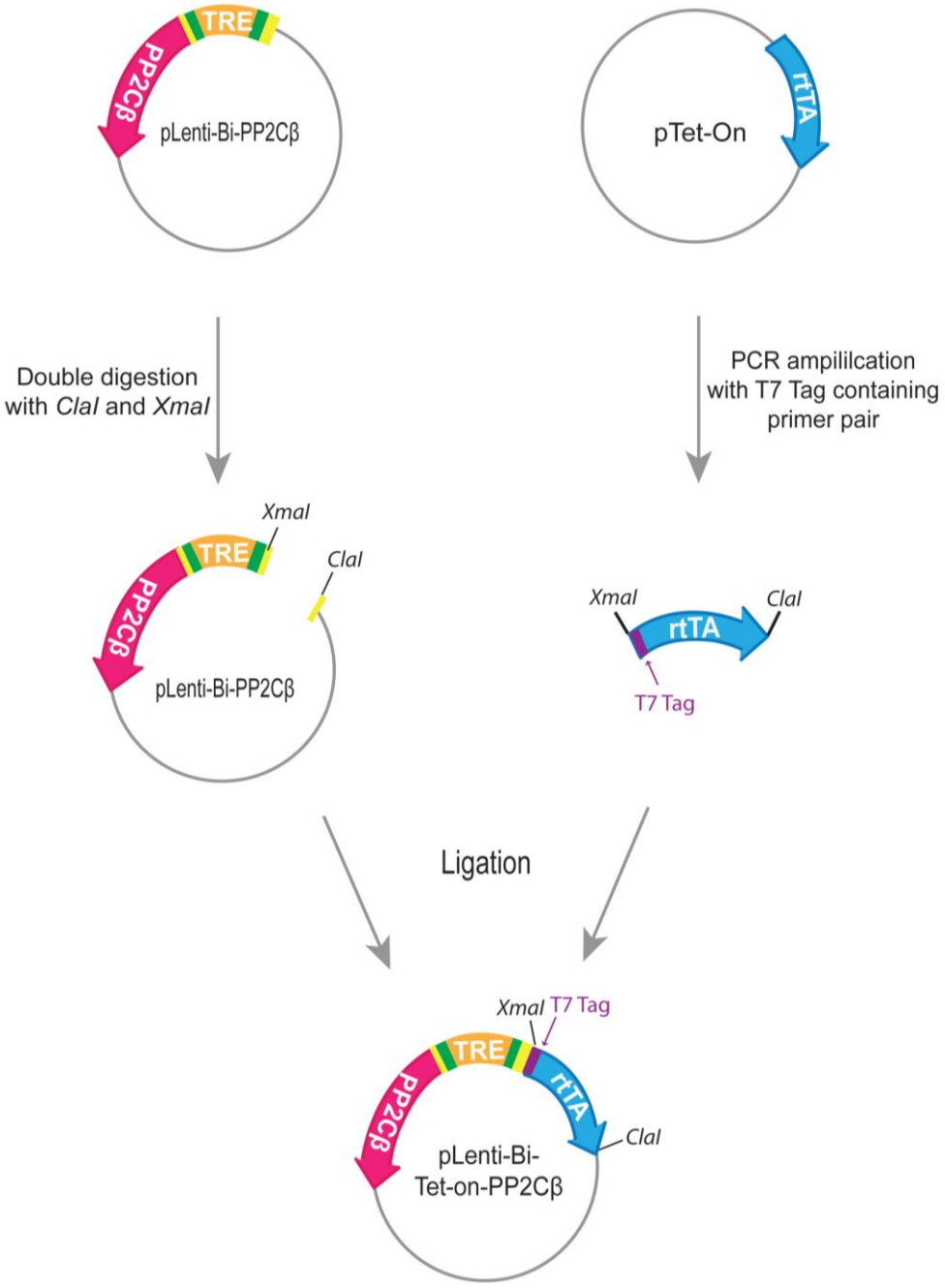

Figure 3.1: Cloning strategy. Bi-directional pLenti-Bi-TRE-PP2Cβ plasmid were linearized using *XmaI* and *ClaI* (Left). The cDNA of the tetracycline-responsive transcriptional activator (*rtTA)* fragment was amplified with a T7-tag-containing primer pair (Table 3.1) using PCR from pTet-on plasmid (Right). This *rtTA* fragment was then inserted into each pLenti-Bi plasmid by ligating at *XmaI* and *ClaI* sites.

Table 3.1: Primers used for *rtTA* fragment amplification.

Primer Name	Primer Sequence (5' → 3')*	Tm (°C)	Appended Sequences
TetOn_Xma_ T7_F	GAC CCC GGG CAC CAT GGC TAG CAT GAC TGG TGG ACA GCA AAT GGG TAT GTC TAG ATT AGA TAA AGG T	71.0	*XmaI* site, T7 tag
TetOn_Cla_R	TCC TCG ATC GAT CTA CCC ACC GTA CTC GTC AAT	64.9	*ClaI* site

*T7-tag: blue, restriction sites: red, random end sequences: underlined.

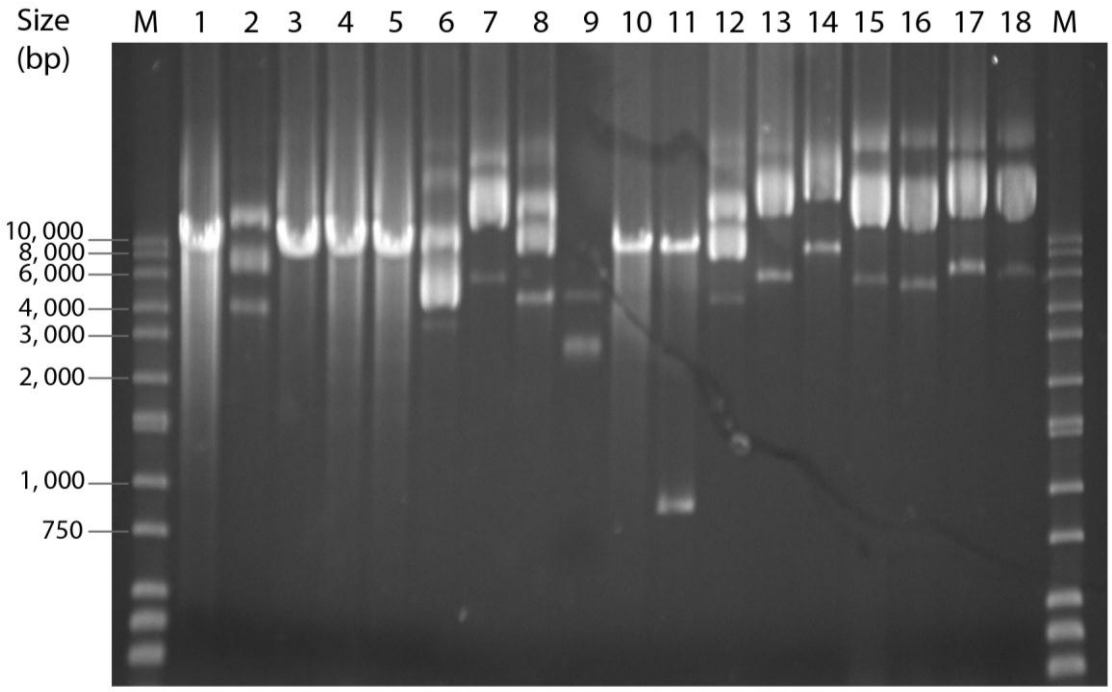

Figure 3.2: Screening of *rtTA* in V and MUT constructs using 1% agarose gel electrophoresis. Electrophoresis was carried out for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. M: DNA molecular weight marker. Lane 1-5: *XmaI* and *ClaI* digestion of candidate plasmid DNA isolated from colonies transformed with ligation reaction for the construction of V plasmid. Lane 6-18: *XmaI* and *ClaI* digestion of candidate plasmid DNA isolated from colonies transformed from MUT construct ligation reaction.

3.1.3 Construction of pLenti-Bi-TRE-Tet-On-PP2Cβ MUT (MUT)

The ligation reactions for pLenti-Bi plasmids [pLenti-Bi-TRE, pLenti-Bi-TRE-PP2Cβ WT and pLenti-Bi-TRE-PP2Cβ MUT] were used for *E. coli* transformation and successful transformants were selected on LB/agar plates containing ampicillin (50 µg/mL). Five colonies containing V and thirteen colonies containing MUT plasmids

were screened by one-step double digestion with *XmaI* and *ClaI*. Only one of the MUT colonies appeared to have an insert corresponding to the molecular weight of the *rtTA* fragment ~1000 bp (Lane 11, Figure 3.2). The size of *rtTA* in MUT construct (Lane 3, Figure 3.3) was identical to that of PCR amplified *rtTA* fragment (Compare lane 1 with lane 3, Figure 3.3). The sequence of *rtTA*, PP2Cβ and the R179G mutation in this MUT construct were confirmed by Sanger sequencing analysis, carried out by the Massey genome service. An additional *XmaI* restriction site was found at the C-terminal end of *rtTA* (Appendix IV and V), therefore, the observed size of *rtTA* fragment on agarose gel was ~900 bp rather than the full length ~1100 bp (Figure 3.3). The plasmid map and the corresponding sequence of *rtTA* and *PP2Cβ* are presented in Appendix IV and V. The *PP2Cβ* primer pair used for sequencing is illustrated in Appendix I.

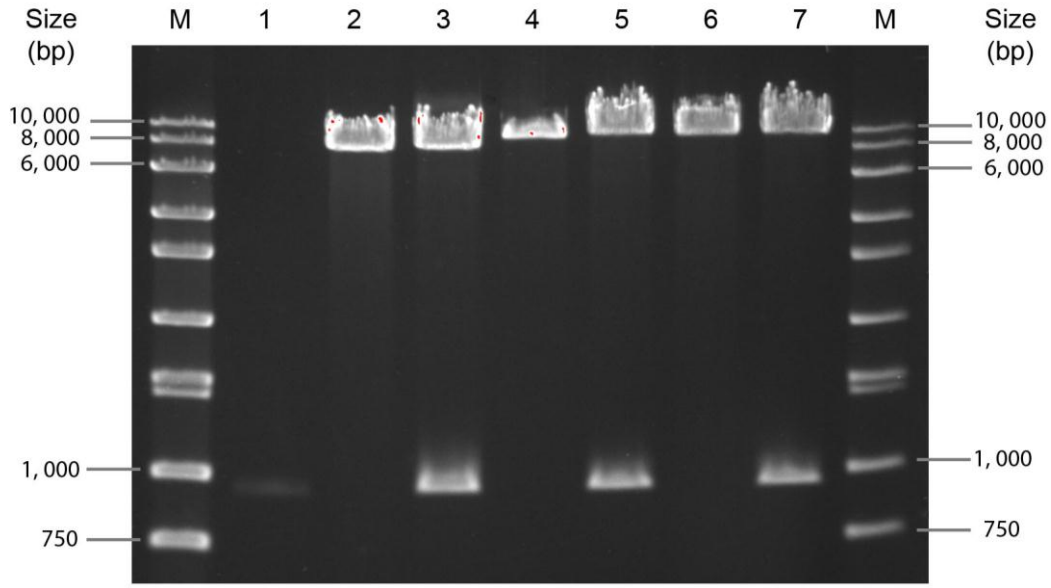

Figure 3.3: Confirmation of *rtTA* containing constructs using 1% agarose gel electrophoresis. Electrophoresis was carried out for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. M: DNA molecular weight marker. Lane 1: positive control, *rtTA* fragment amplified from PCR and digested with *XmaI*. Lane 2, 4, 6: uncut V, WT, MUT constructs. Lane 3, 5, 7: V, WT, MUT constructs digested with *XmaI*. The size of *rtTA* from a restriction enzyme digestion of the candidate plasmid was consistent to that of amplified *rtTA* size ~1000 bp. The construction of V and WT was later performed by different strategy as described in Section 3.1.4.

3.1.4 Construction of pLenti-Bi-TRE-Tet-On (V) and pLenti-Bi-TRE-Tet-On-PP2Cβ WT (WT)

The second *XmaI* site within the cDNA of *rtTA* caused a major problem in the construction of V and WT plasmids. A unique restriction enzyme site other than *XmaI* site at the N-terminal was thought to solve this problem. However, no suitable endonuclease site was found to give a single cleavage in either the N-terminal end of *rtTA* fragment or pLenti-Bi plasmids. Therefore an alternative cloning strategy was developed to accomplish V and WT plasmid construction. A unique *XhoI* restriction site was found on the pLenti-Bi plasmids, which is located upstream of the *PP2Cβ*'s open reading frame (ORF). Most importantly, *XhoI* does not cut cDNA of *rtTA*. Therefore, the second cloning strategy employed *XhoI* and *ClaI* sites to cleave a larger fragment containing *rtTA* from MUT plasmid, instead of using PCR amplified *rtTA* from pTet-On plasmid, and insert this fragment into *XhoI* and *ClaI* digested pLenti-Bi V and pLenti-Bi WT plasmids. However, no *rtTA*-containing DNA fragment was produced from *XhoI* and *ClaI* digested MUT plasmid. To see if *XhoI* site really exists in the MUT plasmid, plasmid DNA was digested with *XhoI* and *ClaI* individually. Surprisingly, digestion with *ClaI* failed to produce a linearized plasmid DNA on agarose gel, possibly due to DNA methylation-mediated inhibition of *ClaI* endonuclease activity. Therefore, cleavage of *rtTA* from *ClaI* site was no longer feasible even though DNA sequencing analysis confirmed the *ClaI* site on the MUT plasmid. The restriction enzyme, *EcoRV*, which will cut adjacent to the 3' end of *ClaI* site, was then selected to substitute *ClaI* site.

The second strategy was employed using *XmaI*, which has been allowed to cleave the 5' end of *rtTA* for a limited time frame for a partial digestion. MUT plasmid was first digested with *EcoRV* for 1 hour, followed by partial digestions with *XmaI* (1:5, 1:10 and 1:20 dilution) under a restricted time frame. The resultant digestion mixtures were analysed on an agarose gel. Digestion with 1:5 diluted *XmaI* was found to produce a full length *rtTA* fragment at a size of ~1100 bp (a, Figure 3.4 and Appendix IV), simultaneously with a fully digested *rtTA* fragment at a size of ~980 bp (b, Figure 3.4 and Appendix IV). In addition, two plasmid bands (~10,000 bp) were also produced by incomplete digestion (Lane 2, Figure 3.4). Due to the high efficacy of *XmaI*, majority of the MUT construct underwent complete digestion. Therefore, a brighter band of smaller

size of *rtTA* band (b, Figure 3.4) was observed compared to the partial digested product (a, Figure 3.4). This upper *rtTA* band were then excised from a preparative agarose gel and ligated into digested pLenti-Bi V and WT plasmids. The correct size of *rtTA* in V and WT constructs were observed on an agarose gel after *XmaI* digestion (Lane 5 and 7, Figure 3.3). The sequences of *rtTA* and *PP2Cβ* in V and WT constructs were also confirmed by Sanger sequencing analysis that was performed by the Massey genome service. Full sequence of the WT plasmid is presented in Appendix V.

In summary, three bi-directional lentiviral plasmids (V, WT and MUT) were successfully constructed by inserting cDNA of *rtTA* derived from the pTet-On plasmid.

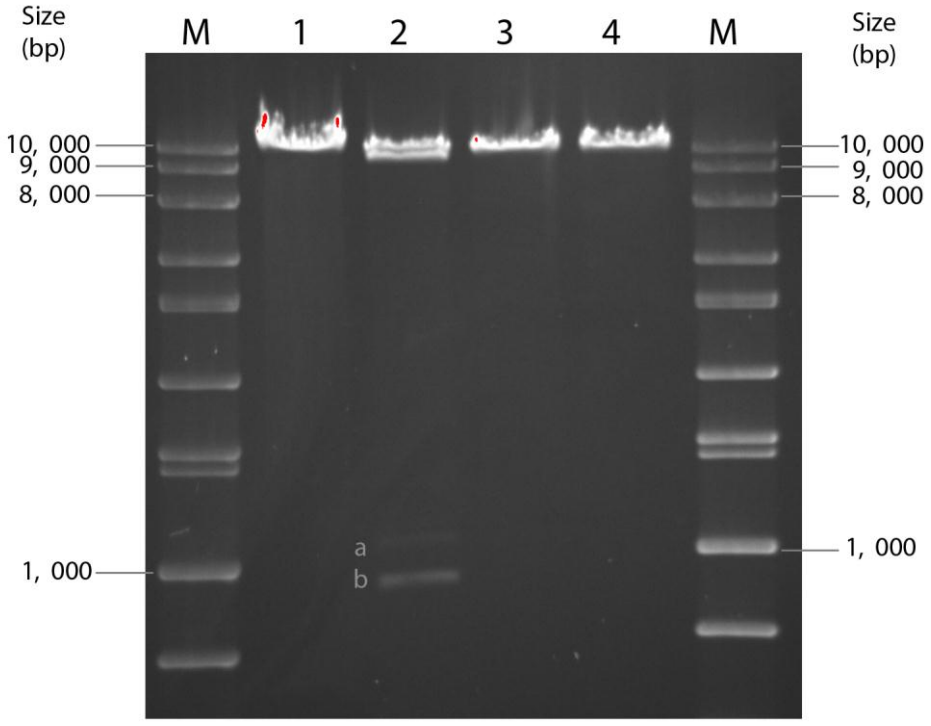

Figure 3.4: Partially digested MUT construct with *XmaI* and *EcoRV* to produce a full length *rtTA* fragment. One representative 1% agarose gel electrophoresis was carried for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. MUT construct was digested with *EcoRV* for an hour to linearize the construct (lane 1), followed by a partial digestion with diluted *XmaI* (1:5, 1:10 and 1:20) under a restricted time frame to produce: **a:** full length *rtTA* with a size of ~1100 bp; **b:** fully digested *rtTA* with a size of ~980 bp. Lane 1: MUT construct digested with *EcoRV*; lane 2-4: *EcoRV*-digested MUT construct further digested with diluted *XmaI* with a dilution of 1:5, 1:10 and 1:20, respectively.

3.2 Confirmation of inducibility of rtTA and PP2Cβ expression in V, WT and MUT vectors.

3.2.1 Introduction

Lentiviral infection requires the packaging from lentiviral vectors into lentiviral particles, followed by a selection process for successful infections. These processes were expected to take several weeks to complete. Therefore, before attempting infection in HEK 293T cells or aged cells, it was deemed necessary to verify that the rtTA and PP2Cβ are expressed in V, WT and MUT vector constructs, and the expression of these two genes only takes place when Dox is presented as an inducer in the cell. When Dox is added into the cell medium, it was hypothesized that minute leaky expression of rtTA in the cell would lead to the formation of an rtTA-Dox complex. This complex is then bound to TRE-element to activate adjacent mini CMV promoters, therefore enhancing the transcription of their downstream genes *rtTA* itself and *PP2Cβ*. Eventually, the elevation of rtTA results in a feed forward loop which simultaneously activates the transcription of *rtTA* as well as *PP2Cβ*. Expression of rtTA and PP2Cβ is expected to be maintained in the presence of Dox.

3.2.2 Confirmation of inducibility in transient transfected HEK 293T cells

V, WT or MUT vector constructs were independently transfected into HEK 293T cells, and after 24 h post-transfection, 1 µM of Dox was added for 24 h induction. A parental, pLenti-Bi vector was also transfected into HEK 293T cells as a negative control. Whole cell protein extracts of transfected HEK 293T cells were prepared. T7 tag is an 11 amino acid peptide encoded in the leader sequence of T7 bacteriophage gene 10, and serves as a tag for easy detection of rtTA production in immuno blotting. Upon Dox induction, V, WT and MUT constructs showed a clear induced expression of T7 tag (Lane 4, 6 and 8, Figure 3.5) compared to no Dox induction (Lane 3, 5 and 7, Figure 3.5). Consistent with the hypothesis proposed in Section 1.4.1, 24 h Dox induction increased the amount of rtTA which was correlated with increased PP2Cβ expression in the WT and MUT constructs (Lane 6 and 8, Figure 3.5). The size of PP2Cβ is around 60 kDa, however, a minor band below 60 kDa was also observed in Lane 6 and 8 in Figure 3.5. This extra band may be caused by PP2Cβ antibody which could recognize more than one PP2Cβ isoforms, as PP2Cβ has six distinct isoforms being produced by

alternative splicing of a single pre-mRNA [46, 47, 50]. However, HEK 293T cells were shown to produce alternative PP2Cβ isoforms at very low quantities. Alternatively, this extra band could result from degradation of PP2Cβ during sample preparation. In this experiment, the empty pLenti-Bi plasmid (V) which does not contain rtTA or PP2Cβ was used as a negative control. As expected, cells transfected with this vector did not show any expression of rtTA and PP2Cβ regardless of Dox induction (Lane 1 and 2, Figure 3.5). Overall, these results confirmed that V, WT and MUT constructs were able to express either rtTA alone or rtTA and PP2Cβ together. These results also demonstrated that the expression of rtTA and PP2Cβ was dependent on Dox in these vectors.

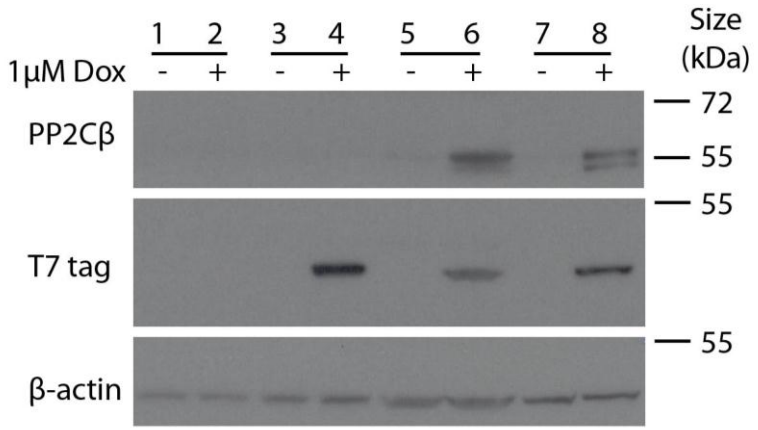

Figure 3.5: Confirmation of Dox-dependent rtTA and PP2Cβ expression via transient transfection on the immunoblot. Western blot analyses were performed on 10% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox inductions were performed on >80% confluent HEK 293T monolayers for 24 h. Equal amount of HEK 293T protein extracts were prepared, and 100 µg of each sample were loaded on the gel. β-actin, a size of ~42 kDa, was included as a loading controls. Lane 1, 2: plenti-Bi, uninduced and induced; lane 3, 4: plenti-Bi-Tet-On (V), uninduced and induced; lane 5, 6: plenti-Bi-Tet-O-PP2Cβ WT (WT), uninduced and induced; lane 7, 8: plenti-Bi-Tet-O-PP2Cβ MUT (MUT), uninduced and induced.

3.2.3 Optimization of Doxycycline concentration

In order to achieve long term stable expression of the transgene under the control of the Tet-On system, the inducer molecule, Dox, must be administered regularly. However, regular administration of an antibiotic can lead to adverse side effects such as loss of

efficacy and drug resistance. Therefore, it was essential to determine the optimal amount of Dox (ideally lowest amount) and the best dose-response time that is required for induction, in order to reduce the possible adverse effects.

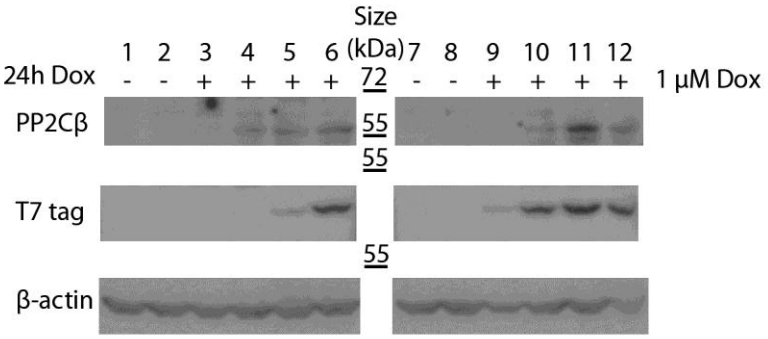

Figure 3.6: Determination of the minimum Dox concentration and dose-response time in HEK 293T cells transfected with MUT construct. Western blot analyses were performed on 10% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox inductions were performed on >80% confluent HEK 293 monolayers in a dose-response (left panel) or a 48 h time course (right panel). Equal amounts of HEK 293T protein extracts were prepared and 100 µg of each sample were loaded. β-actin was included as a loading controls, with a size of ~42 kDa. Lane 1 and 7: Non transfected HEK 293T cell extracts, negative control; lane 2 and 8: HEK 293T transfected with MUT construct, uninduced; lane 3-6: HEK 293T transfected with MUT construct, induced with 1 nM, 10 nM, 100 nM and 1 µM of Dox for 24 h, respectively; lane 9-12: HEK 293T transfected with MUT construct, induced by 1 µm of Dox for 6 h, 12 h, 24 h and 48 h, respectively.

A MUT construct containing both rtTA and PP2Cβ expression was selected for this set of optimization experiments. HEK 293T cells were transiently transfected with the MUT construct. The target gene expressions were examined according to either a dose response manner (Left panel, Figure 3.6) or a 48 h time-course response (Right panel, Figure 3.6), followed by western blot analyses. In the dose response experiment, transfected cells induced by 100 nM and 1 µM of Dox administration for 24 h showed a band in T7 tag western blot (Lane 5 and 6, Figure 3.6) but faint T7-rtTA bands could be detected by Dox lower than 100 nM (Lane 3and 4, Figure 3.6). Based on signal intensity, a stronger rtTA T7-rtTA expression appeared to be induced by 1 µM of Dox treatment compared to treatment with 100 nM of Dox. Due to the endogenous expression of PP2Cβ, a basal level of PP2Cβ expression was observed in all lanes. However, Dox concentrations at 10 nM, 100 nM and 1 µM clearly induced PP2Cβ

expression above the basal level (Lane 4, 5 and 6, Figure 3.6). Taken together, these results indicate that the optimal Dox concentration to induce the expression of rtTA and PP2Cβ in MUT was 1 μM. In the time-course experiment (Right panel, Figure 3.6), transfected HEK 293T cells were treated with 1 μM of Dox for a 48 h time course (6 h, 12 h, 24 h and 48 h). Induced T7-rtTA expression was observed in all Dox induced cells (Lane 9-12, Figure 3.6). The induced expression of PP2Cβ could only be observed after 12 h of Dox (1 μM) treatment (Lane 10, 11 and 12, Figure 3.6). Cells treated with 1 μM of Dox for 24 h appeared to have the greatest induction in both T7 tag and PP2Cβ. These results indicate that the optimal dose-response time with 1 μM of Dox is 24 h. Thus, to minimise Dox dosage and maximise induction efficiency, 1 μM of Dox was administered for at least 24 h in subsequent experiments. These induction conditions were thought to be the same for V and WT vectors because they were constructed in a similar way as the MUT vector.

3.3 Inducibility of lentivirally infected HEK 293T cells

3.3.1 Introduction

Since the inducibilities of rtTA and PP2Cβ in three vector constructs (V, WT and MUT) were confirmed in transient transfected HEK 293T cells using Dox treatment, the inducibilities of rtTA and PP2Cβ in lentivirally infected cells was examined. Given that HEK 293T cells are fast growing and easy to work with, Dox inducibility in lentivirus-infected HEK 293T cells was investigated before attempting primary cell lines. The packaging cell line HEK 293T was used to prepare lentiviral particles from the lentiviral vectors of V, WT and MUT.

3.3.2 Optimisation of lentiviral packaging

Three plates of HEK 293T cells were set up for lentivirus packaging. Individual transfer vectors of V, WT or MUT were independently co-transfected into HEK 293T cells with packaging plasmids. After co-transfection, lentiviral packaging is known to take place inside of HEK 293T cells and be released into the culture medium. After three days of co-transfection, viral particles in the culture medium were harvested, purified and concentrated to produce lentivirus stock (Section 2.2.3).

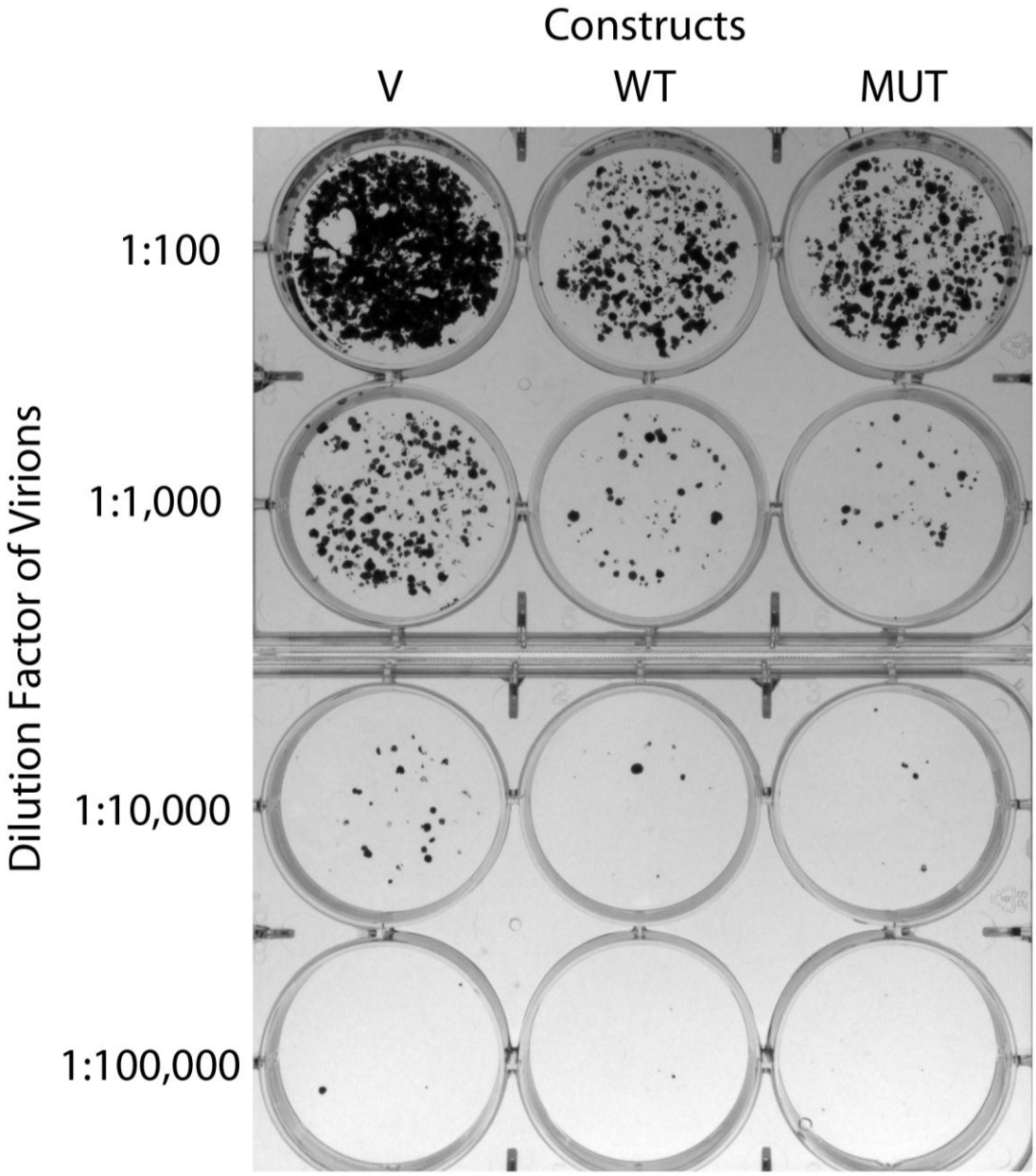

Figure 3.7: Colony formation of lentivirally infected HEK 293T cells. A density of 1×10^5 per well of HEK 293T cells were seeded and infected by lentivirus according to 10-fold serial dilutions (1:100, 1:1,000, 1:10,000, 1:100,000). Infected cells were selected with puromycin selection medium, consisted of DMEM supplemented with 10% FBS, 0.5% Pen/Step, 1-2 µg/mL Puromycin, for 10 days followed by crystal violet staining. Colonies in each well were counted and viral titers were calculated according to the formula, #colonies/2 mL medium×dilution factor = CFU/mL viral stock.

Serial dilutions of these lentivirus stocks were used to determine the optimal viral titer for each lentivirus. HEK 293T cells (1×10^5 cells) were seeded onto each well of two 6-well plates and infected by lentivirus according to 10-fold serial dilutions (1:100, 1:1,000, 1:10,000, 1:100,000; Figure 3.7). After infection, all wells were subject to

puromycin selection for approximately 10 days, until colonies were established in each well. Cells were stained with crystal violet for visualization, and colonies were counted and calculated to generate a viral titer for each lentivirus stock. After 10 days of puromycin selection, 1:10,000 and 1:100,000 dilution of each virus displayed almost zero or very low level of HEK 293T survival rate, respectively. Cells infected with V lentivirus appeared to be 5-6 times more efficient, with more colonies observed in each dilution, than that of WT and MUT viruses (Table 3.2). These results together suggest that three lentiviruses were successfully prepared and all of them have a reasonable ability to infect HEK 293T cells, although the infection efficiency is variable between different constructs.

Table 3.2: Viral titer of the three lentviruses.

Lentivirus	Viral Titer (CFU/mL*)
pLenti-Bi-TRE-Tet-on	~8.6×10^4
pLenti- Bi-TRE-Tet-on-PP2Cβ WT	~1.5×10^4
pLenti-Bi-TRE-Tet-on-PP2Cβ MUT	~1.6×10^4

*CFU: colony-forming unit.

3.3.3 Stability of PP2Cβ in response to Dox induction

The stability of PP2Cβ in response to Dox induction in lentivirally transduced HEK 293T cells was examined to assess the feasibility of this lentiviral Tet-On system. A functional lentiviral Tet-On system requires the ability to induce rtTA and PP2Cβ expression upon Dox induction (On state) and the ability to return to basal level when Dox is removed from the system (Off state). Three plates of lentivirally transduced HEK 293T cells for each lentivirus (V, WT and MUT, vector amount was used according to viral titer in Table 2.1) were cultured and selected until ~80% confluency was obtained. Dox (1 µg/mL) was added into two of these plates (On state) and no Dox was added to the remaining plate as a negative control. The untreated plate and one of the Dox-treated plates were harvested after 24 h of Dox treatment, while the remaining Dox plate was further split into three plates and cultured with no Dox medium for another 24 h, 48 h and 72 h (Off state). Ideally, removal of Dox from the culture medium will result in inactivation of the system (Off state). Western blot analyses were

carried out to examine the stability of rtTA and PP2Cβ in response to the presence and absence of Dox (On vs Off states). However, neither rtTA nor PP2Cβ expression was observed in any of lentivirally infected HEK 293T cells regardless of Dox induction (Data not shown).

This unexpected result could be due to several factors. For instance, the infection efficiency was too low to give a reasonable amount of gene expression. To solve this problem, viral infection was attempted in 24-well plate with the application of more viral particles under puromycin selection until ~80% confluence in a 6 cm tissue culture plate was obtained. Two plates were set up for each lentivirus, one of them were teated with Dox for 24 h before harvest. A negative control, uninfected HEK 293T cells, and a positive control, transfected HEK 293T with V construct were also included in the western blot analyses. Once again, neither rtTA nor PP2Cβ expression was observed upon Dox induction. The increase in viral titer applied to the target cells was expected to increase the infection efficiency, leading to more effective gene induction. However, no induction was observed after three repeats. These negative results showed that virally infected HEK 293T cells were not able to induce rtTA and PP2Cβ regardless of Dox in the system. Given that cells were successfully infected and survived from puromycin selection as demonstrated in Figure 3.7, transduction into a host chromosome seems to affect the Dox inducibility, which was otherwise observed in the transient transfection method (Figure 3.5). Taken these results into consideration, these experiments suggest some difficulties for Dox-dependent instant induction of this lentiviral Tet-On system.

3.3.4 Optimisation of lentiviral induction condition

Since it takes a long time to establish confluent infected cells before Dox induction, an alternative induction strategy was developed. Viral titer experiments showed that HEK 293T cells infected by V virus had 5-6 times higher infection titer than those infected by WT or MUT viruses. Therefore, V virus was used to optimize conditions for lentiviral induction.

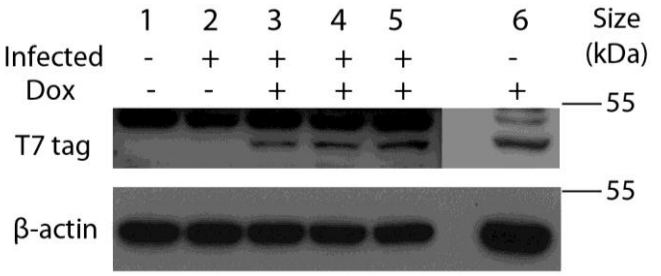

Figure 3.8: Time course of rtTA expression in response to Dox induction using V virus infected HEK 293T cells. Western blot analyses were performed on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox induction was performed in a time course by adding Dox, 24 h, 48 h and 72 h after infection. Equal amounts of infected HEK 293T extracts were prepared after cells are confluent, and 20 µL of each sample were loaded on the gel. β-actin was included as a loading control. Lane 1: Uninfected HEK 293T, negative control; lane 2: infected HEK 293T with no Dox; lane 3-5: infected HEK 293T with Dox induction after 24 h, 48 h and 72 h of infection, respectively; lane 6: positive control, HEK 293T cells transiently transfected with V construct, induced with Dox.

HEK 293T cells were plated and infected in a 24-well plate as described in Section 3.3.3, Dox was added after lentiviral infection in a time course manner, 24 h, 48 h and 72 h post-infection (Lane 3, 4 and 5, respectively Figure 3.8). Dox was maintained in the system until the infected cells reached >80% confluency in a 6 cm plate. Cells were harvested for western blot analysis. Parental HEK 293T cells and transiently transfected HEK 293T cells with V construct were included as a negative and positive control, respectively (Lane 1 and 6, Figure 3.8). Due to a high induction efficiency and the higher cell number (Lane 6 in β-actin panel, Figure 3.8), the positive control sample was developed separately for a shorter time to avoid an intensive signal on a detection film. Consistent with T7-rtTA expression of the positive control (Lane 6, Figure 3.8), virally infected HEK 293T cells with Dox induction showed a band at the same position of T7-rtTA (compare Lane 6 with Lane 3, 4 and 5, Figure 3.8). The addition of Dox 72 h post-infection appeared to have the strongest T7 tag expression (Lane 5, Figure 3.8) compared to the band at 24 h and 48 h (Lane 3 and 4, Figure 3.8) suggesting that lenvirial infection followed by immediate Dox induction improves inducibility of the system. However, this result could not be consistently reproduced using the same cells, suggesting that rtTA was expressed at low levels below the sensitivity threshold of the detection method. In summary, these results showed that V virus infected HEK 293T

cells can be induced by adding Dox within 72 h of post-infection, however, the induction of this system is not efficient enough for a reproducible detection by immunoblot analysis.

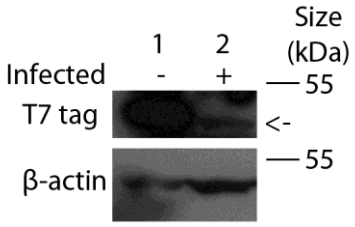

Figure 3.9: Examination of rtTA protein expression in response to Dox induction in stably established V lentivirus-infected HEK 293T cells. Western blot analysis was performed on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 130 V and transferred for 2 h at 150 mA onto a PVDF membrane. Dox induction was performed on stable cell line over 72 h. Lane 1: positive control, HEK 293T cells transiently transfected with V construct, induced with Dox; lane 2: V stable cell line, induced with Dox for over 72 h.

3.3.4.1 Induction in stable cell line

The above studies (Section 3.3.3 and 3.3.4) demonstrated that the inducibility of this Tet-On lentiviral system is not very stable in an infected polyclonal cell population. Individual stable cell lines for all three constructs derived from a single colony were produced and examined for Dox inducibility. HEK 293T cells were plated and infected in 6-well plates as described in Section 3.3.2. Cells were transduced by each virus independently with a 10-fold serial dilution and selected with puromycin until single colonies were developed. Three colonies were picked for each virus and cultured until they became confluent in 6 cm tissue culture plates. Dox treatment was performed for more than 72 h, followed by western blot analysis. One of V lentivirus-infected stable cell lines exhibited weak T7-rtTA expression after Dox induction in which the band matched to the T7-rtTA from transiently transfected positive control cells (compare Lane 2 to Lane 1, Figure 3.9). Cell extract of stable cell line was intentionally loaded more than that of the positive control to visualize a weak expression of T7-rtTA, and therefore β-actin from the stable cell line is more intense than that in positive control. Negative controls from parental HEK 293T cells and V lentivirus-infected stable cell line without Dox, were performed on another gel and did not show any signal of T7-rtTA (Data not shown). Nevertheless, other stable cell lines failed to produce an induced

expression of the T7 tag by Dox. In conclusion, the induced T7 tag expression in V infected stable cell lines was not reproducibly observed every time. This result confirmed that V lentivirus-infected HEK 293T cells could be induced by addition of Dox, but illustrated a instability and inconsistency of this induction system.

3.4 Examine *rtTA* expression using reverse transcriptase-coupled quantitative real-time PCR

3.4.1 Introduction

Reverse transcriptase-coupled quantitative real-time PCR (RT-qPCR) is one of the most sensitive methods for mRNA detection and quantification. This sequence-specific procedure monitors the relative quantities of reverse transcribed mRNA after each PCR cycle in real time which has become a standard method for transcription analysis. HEK 293T cells transiently-transfected with the V construct displayed increased protein expression of rtTA in response to Dox treatment (Figure 3.5). However, lentivirus-mediated infection and subsequent probing by immunoblotting was inconsistent with the Dox induced protein expression of rtTA. This inconsistency could be due to weak protein expression or ineffective Dox induction. Therefore, in order to validate the inducibility in virally infected cells, RT-qPCR was employed to detect the changes in *rtTA* expression in response to Dox treatment at the mRNA level.

3.4.2 Primer design

The first step of RT-qPCR was designing primer pairs for the target gene (*rtTA*) and reference genes. Reference gene Beta Glucuronidase (*GUSB*) primer pair for RT-qPCR analysis was obtained from Qiagen (QuantiTect Primer Assay, catalog number: QT 00046046). Three *rtTA* primer pairs were designed based on the pTet-On plasmid sequence from Clontech, with amplicon sizes of 68 bp, 186 bp and 182 bp (Table 3.2). Intra- and inter-primer complementarities were monitored by the probe design software (Roche) and thus avoided in final primer design.

Table 3.3: Sequences, melting temperatures (Tm ºC) and amplicon size of *rtTA* primer pairs.

Primer Pair	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Tm (°C)	Size (bp)
1	TAATGAGGTCGGAATCGA	TAGGCTGCTCTACACCAAG	60	68
2	TAGATGTGCTTTACTAAGTCATC	GATCTTCCAATACGCAACCTA	60	186
3	CACTTAGACGGCGAGGAC	ATTCCAAGGGCATCGGTA	60	182

The specificity of amplification by each primer pair and amplicon sizes were examined using conventional PCR and visualized on a 2% agarose gel (Figure 3.10). One amplification product was observed for each prime pair (Figure 3.10) with the expected size as indicated in Table 3.3. Negative controls containing no cDNA template were also tested with no visible bands detected (Data not shown). This indicates that each amplicon was specifically produced by the corresponding primer pair rather than by a random non-specific amplification.

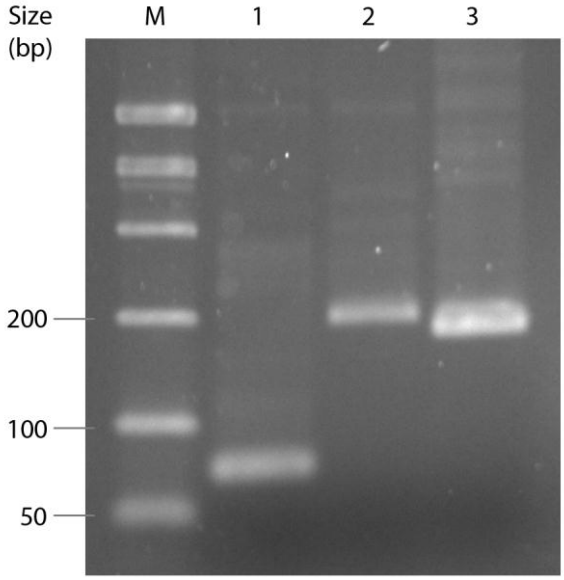

Figure 3.10: Specificity check of *rtTA* amplicon using a conventional PCR. Agarose gel electrophoresis (2%) was carried for 1 h at 120 V in 0.5 × TBE buffer and stained with 0.5 µg/mL ethidium bromide. Ten percent of each 50 µL PCR reactions was loaded into a well and was subject to gel electrophoresis. Lane 1, 2, and 3: PCR reactions using the *rtTA* primer pairs 1, 2 and 3, respectively. M: molecular weight marker. Negative controls containing no cDNA template were carried out on a separate gel with no detection of any bands (Data not shown).

3.4.3 RNA quality control

High quality RNA preparation is essential in RT-qPCR analysis. Approximately, the same quantity of RNA should be used for each sample for relative quantification by RT-qPCR. Therefore, accuracy of RNA quantification is crucial to generate reliable data. Quantity, purity and integrity are three quality control steps that are normally performed on RNA samples. Spectrophotometry was utilised in this study for RNA quality control using the Nanodrop® ND-1000 spectrophotometer.

DNase treatment was carried out to eliminate genomic DNA contamination using 30 µg of extracted RNA according to the concentration determined by spectrophotometry (Table 3.4, "before" column). RNA concentrations determined using the Nanodrop spectrophotometer, of each sample before and after DNase treatment are listed in Table 3.4. Optical density (OD) of 260/280 and 260/230 ratios were used to assess RNA purity before and after DNase treatment Table 3.4. After DNase treatment, $OD_{260/280}$ ratios for each RNA sample fell into the acceptable range, but most of $OD_{260/230}$ ratios were detected below the 1.8 cut off mark regardless of DNase treatment. These results suggest that all RNA samples were extracted with relatively high purity, however, traces of salt or solvent contamination might be present in RNA samples.

Table 3.4: Camparison of RNA concentration before and after DNase treatment using Nanodrop spectrophotometry.

Sample	Before DNase Treatment			After DNase Treatment		
	Conc. (ng/µL)	$OD_{260/230}$	$OD_{260/280}$	Conc. (ng/µL)	$OD_{260/230}$	$OD_{260/280}$
HEK 293T	476.33	0.99	1.97	296.58	0.93	2.07
I-VI -Dox*	514.10	1.76	1.97	425.91	1.4	2.07
I-VII +Dox	861.70	1.90	2.02	557.37	1.75	2.03

* I: V lentivirus-infected cell line

3.4.4 Reverse transcriptase negative control

The reverse transcription step introduces substantial errors into RT-qPCR analysis, therefore, it is essential to eliminate the potential interference produced through

contamination by genomic DNA. This study utilized a one-step RT-qPCR analysis that uses RNA samples to produce reverse transcription and PCR amplification together in the same tube. For each sample, a reverse transcription negative control (RT-) was run in parallel whereby the reverse transcriptase was omitted. In general, detection after 35 amplification cycles is considered to be close to background. No detection of amplification (or Crossing point (Cp) value > 40 cycles) was observed in RT- samples, indicating that these samples did not have detectable levels of genomic DNA contamination.

3.4.5 PCR efficiency optimization

High PCR efficiency assures robust and accurate RT-qPCR results, especially when relative quantification analysis is involved. Target and reference genes are required to have comparable high amplification efficiencies to allow accurate relative comparison. PCR amplification efficiencies were initially determined by preparing standard curves using the Lightcycler® 480 system. A 10-fold serial dilution series (1:5 to 1:500) of an RNA sample was prepared in triplicate, in order to construct a standard curve for each target and reference gene (Appendix VII-VIII). Crossing points (Cp or Ct) for amplification using each primer pair were collected and plotted against the Log_{10} concentration of each dilution to generate a standard curve. PCR amplification efficiencies were then calculated from the slopes of the standard curve according to $Efficiency = 10^{-1/slope} - 1$ [81]. Theoretically, the quantity of PCR product should double with each amplification cycle, therefore, the maximum efficiency of a PCR amplification is 2 [81]. Nevertheless, experimental procedures may introduce errors to PCR amplification, with the range of efficiencies between 1.9 and 2.1 (5% error) being generally accepted. The *rtTA* primer pair 2 and GUSB primer pair showed reasonably high PCR efficiencies of 2.04 and 1.97 (Appendix VII-VIII), respectively. These efficiencies fall into the acceptable range, and herefore suitable for subsequent analysis. Almost all sample dilutions shared the same melting peak when amplified using the same primer pair, indicating that the primers were highly specific. Together with the acceptable efficiency, *rtTA* and *GUSB* are suitable for subsequent analyses to monitor inducibility of *rtTA*. The melting curve of *rtTA* at 1:1000 dilution exhibited a shoulder peak around 72°C. This was caused by the formation of primer dimer which was

confirmed by running PCR amplification product on a 2% agarose gel (Data not shown).

3.4.6 Relative quantification

Since the inducibility of rtTA protein level was unstable in V infected HEK 293T cells and hard to be detected in western blotting, real-time quantitative PCR (RT-qPCR) was used to detect induced mRNA level of *rtTA*. Dox treatment was performed 24 h post-infection, and maintained in the system until the infected cells reached >80% confluency in a 10 cm plate. Cells were harvested for western blot and RT-qPCR analyses. Western blot analysis of V virus-infected HEK 293T cells showed a faint T7-rtTA band when induced with Dox (Lane 5, Figure 3.11A) compared to the un-induced sample (Lane 4, Figure 3.11A). Subsequently, RT-qPCR was carried out to confirm this inducibility. Due to the low reproducibility of this system, only one biological replicate was used to carry out RT-qPCR analysis. Three reverse transcription replicates were carried out and relative induced values (Dox) were calculated compared to the untreated control (no Dox) after normalization against reference gene expression. All samples were assessed in triplicate. Dox induced samples showed 2 Ct cycle difference on average from uninduced control, which correspond to an approximate eight fold increase in *rtTA* expression (Figure 3.11B). Due to time constraints, untreated control (no Dox) samples were derived from induced cells by removing Dox from the culture medium for over 72 h. This is not an ideal untreated control, which ideally should be derived from infected cells never treated with Dox. Thus, it is possible that traces of Dox residue in the untreated control may result in a higher basal level of expression compared to an ideal untreated control. Parental HEK 293T samples were also analyzed using RT-qPCR as a negative control, which showed an average Ct value of 32, and therefore deemed to be a background level of RT-qPCR assays. Two-tailed Student's t test was performed with a p-value < 0.001 (asterisk, Figure 3.11B), suggesting that this 8 fold increase in *rtTA* expression is statistically significant in Dox induced cells than that of un-induced cells. Taken together, these results confirmed that *rtTA* can be induced in V lentivirus-infected HEK 293T cells. However, primer dimer interference was found in some samples by visualizing RT-qPCR products on a 2% agarose gel (Data not shown). Extra bands around 50 bp and 40 bp for *rtTA* and *GUSB* amplicons, respectively, were observed which correspond to the size of their primer dimers.

Therefore, the reliability of these assay results needs to be further confirmed from new primer sets that do not produce primer dimer.

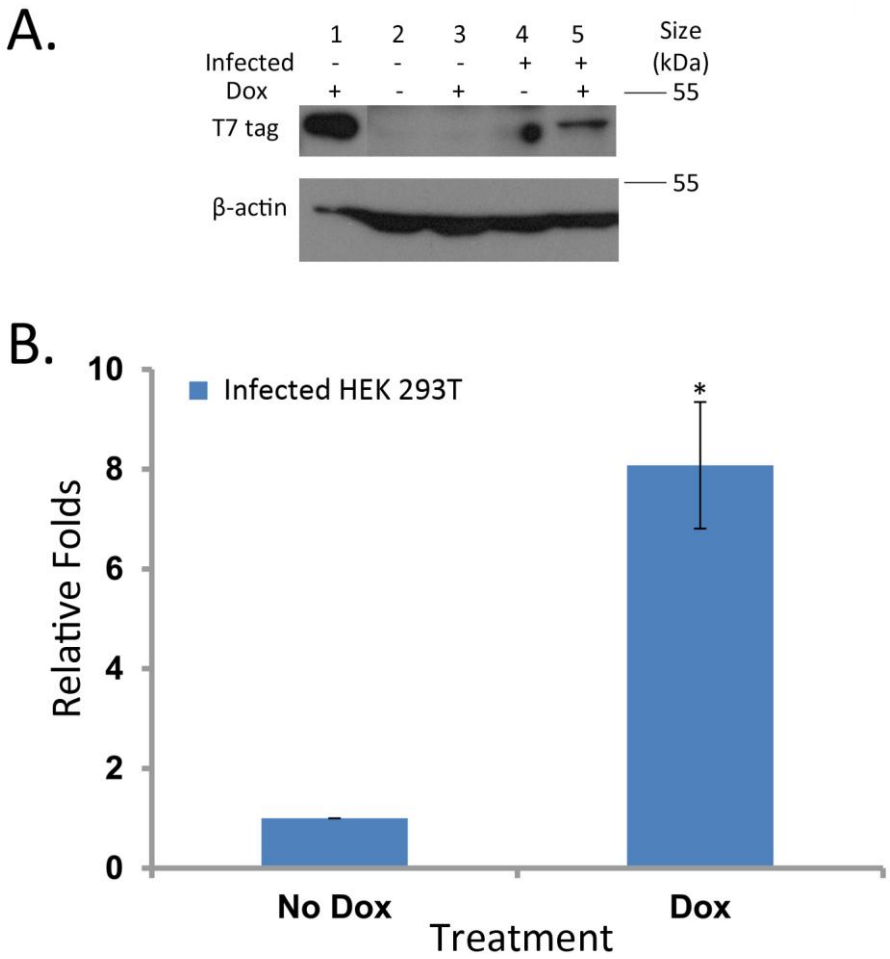

Figure 3.11: Confirmation of induced *rtTA* expression using quantitative real-time PCR analysis. Dox induction was performed 24 h post-infection. **(A)** Western blot analysis was used to detect rtTA protein expression in response to Dox treatment on 8% SDS-polyacrylamide gel electrophoresis for 1.5 h at 135 V and transferred for 2 h at 150 mA onto a PVDF membrane. Positive control (Lane 1) was separately exposed for a shorter time frame to avoid an intensive signal on detection film. Lane 1: HEK 293T cells transiently transfected with V construct, induced with Dox; lane 2-3: HEK 293T cells uninduced and induced with Dox, respectively; lane 4-5: V virus infected HEK 293T, uninduced and induced with Dox, respectively. (B) Relative quantification of gene expression changes in *rtTA* expression in response to Dox induction. Three reverse transcription repeats were carried out for each samples and each sample was tested in triplicate. Dox induced samples exhibited ~8 fold increase in rtTA expression compared to uninduced samples. P-values, calculated by Student's t-test, are presented by asterisk (*: $p<0.001$).

Chapter 4: Discussion and future directions

Lentiviral vector delivery systems are one of the most efficient ways to transfer a gene of interest into target cells, tissues and organs [4, 82]. These vectors have undergone a number of improvements over many years [3, 5, 7, 16]. A target vector is commonly used in combination with a regulatory component such as tetracycline responsive transcriptional activator or repressor to allow tight regulation of the transgene[29, 82-84]. Almost all inducible lentiviral systems have been constructed using at least two vector components, one harbouring the target transgene and the other carrying the transcriptional regulator inducibly acting on the regulatory promoter element[32, 83]. To improve a conventional lentiviral vector with an inducible gene expression feature, an instantly inducible gene expression system that combines the target gene and transcriptional regulator on a single vector was designed and constructed in this study for investigating gene function *in vivo*.

PP2Cβ is associated with a number of pathways including cell proliferation, differentiation, senescence and apoptosis [1, 48, 52, 55, 57]. A recent study revealed a down-regulation of PP2Cβ during cellular senescence in human diploid fibroblasts, suggesting its role in cellular ageing process (unpublished result, Dr. Jeong Park). Here, a novel tetracycline inducible lentiviral vector that expresses both PP2Cβ and a regulatory component (rtTA) on a single plasmid was designed, constructed and characterised to provide a novel tool for future investigating the role of PP2Cβ in the NFκB ageing pathway.

4.1 Construction of lentiviral vectors

For many years, effective lentiviral gene transfer with an inducible gene expression feature was undertaken by placing a gene of interest and a regulatory component on separate plasmids [32, 83]. However, such separated systems proved cumbersome due to difficulties in establishing conditions for effective and precise control of gene expression. Markusic *et al.* was one of the first research groups to incorporate the Tet-On regulatory system into a lentiviral vector as a single cassette and compared the rtTA expression driven by an autoregulatory loop to the one by a constitutive promoter [31]. Since rtTA expression by autoregulatory loop turns out to provide a better lentiviral

vector system [31], this current study made several upgrades to an existing lentiviral vector, allowing more efficient manipulation of the transgenes *in vivo*. Key features of this improved system include the incorporation of (1) a bi-directional lentiviral vector, allowing transcription of two downstream genes simultaneously; (2) a tetracycline response element (TRE) flanked by two minimal CMV promoters, enabling doxycycline-inducible expression of lentivirally delivered transgene in target cells; (3) a regulatory component, rtTA, facilitating inducible and controlled expression of both the gene of interest and rtTA, and (4) a puromycin marker for selection of successfully infected cells.

In this study, three instant Dox-inducible lentiviral vectors, that inducibly express the protein phosphatase PP2Cβ wild type (WT), mutant (MUT) and no PP2Cβ control (V), were successfully developed using a single cassette (Figure 3.3). The original cloning strategy used to generate these vectors involved independent ligation of the *rtTA* fragment obtained by PCR into each of the bi-directional pLenti-Bi vectors (Figure 3.1). Ideally, restriction endonucleases used to cut lentiviral vector should not cut the cDNA of interest (rtTA) for cloning. However, the restriction endonuclease *XmaI* was found to cut inside of the cDNA of *rtTA*, which caused a time-consuming delay in construction of the V and WT constructs. Only the MUT construct was successfully constructed using this strategy. The success of this MUT construction was a particularly fortunate event, because one of MUT candidate colonies was generated from a partial digest of the cDNA of *rtTA*, which gave the full length *rtTA* fragment at a size of ~1100 bp. However, complete digestion of the cDNA fragment of *rtTA* with *XmaI* would yield a truncated *rtTA* fragment (~980 bp) which cannot be used for cloning. Thus, an alternative strategy was attempted, whereby the full size *rtTA* (~1,100bp) containing fragment was obtained by *XhoI* and *ClaI* digestion of the MUT vector. Unfortunately, the *ClaI* site appeared to be methylated in the MUT vector, which prevented *ClaI* from cutting at its recognition sequence. Therefore, the *ClaI* site was substituted by an *EcoRV* site at the 3'-terminal end of the cDNA of *rtTA*. The cDNA of *rtTA* was finally produced from the MUT construct using a time-controlled partial digestion with *XmaI* and *EcoRV* (Figure 3.4), and was subsequently sub-cloned into V and WT bi-directional lentivectors.

4.2 Inducibility of lentiviral vector system

Construction of these pLenti-Bi-TRE-Tet-On-PP2Cβ vectors is expected to allow controlled PP2Cβ expression for future studies to investigate its role in molecular ageing. This novel Tet-On inducible lentiviral system provides a number of benefits compared to traditional binary constitutive systems: (1) it enables one step auto-regulation of the transgene and rtTA by Dox; (2) it avoids the cumbersome prerequisite of constructing a cell line for inducibility because this system combines all elements into one single cassette; (3) it does not require co-transduction, selection or screening, unlike previous binary systems, thus allowing it to be used for *in vivo* applications; and (4) it offers tight regulation of rtTA expression through an auto-regulatory loop, allowing it to minimize the toxic effects associated with traditional systems in which high levels of constitutively expressed rtTA often cause toxicity *in vivo*.

In this bi-directional lentiviral delivery system, rtTA serves as a transcriptional activator in response to Dox. In the absence of Dox induction, expression of PP2Cβ should remain at low basal levels while in the presence of Dox, leaky expression of free rtTA forms a complex with Dox which associates with the TRE element to enhance the transcription of both *PP2Cβ* and *rtTA* (Figure 1.5). The inducibility of this Tet-On lentiviral vector system was confirmed using a transient transfection reaction into HEK 293T cells in which significant induction of rtTA and PP2Cβ protein expression levels were observed in transfected HEK 293T cells upon 1 μM of Dox treatment for 24 h (Figure 3.5). In addition, a mild induction of PP2Cβ and rtTA expression with Dox treatment was observed at a concentration as low as 10 nM, suggesting that the system is highly sensitive to Dox in transiently transfected HEK 293T cells (Figure 3.6). These results also suggest that this novel Tet-On inducible bi-directional lentiviral vector has the ability to manipulate expression of PP2Cβ inducibly, and therefore can be used as an inducible mammalian expression plasmid without viral packaging.

Lentiviral delivery systems have been recognised for their efficient gene transfer into diverse target cell lines [4, 28, 31, 85, 86]. Previously, lentiviral vectors with rtTA expression driven by an autoregulatory loop have been shown to produce higher viral titer and better rtTA inducibility compared to those controlled by a constitutive promoter [31]. Studies by Centilivre *et al.* further support this observation; they

demonstrated in mice that more efficient induction of GFP expression occurred when its expression was driven by an autoregulatory loop versus a constitutive CMV promoter [28].

Once a lentivirus delivers a transgene into HEK 293T cells by infection, the transgene is integrated into the cell genome, allowing it to be stably expressed. However, similar Dox sensitivity and the induced expression in transient transfected cells could not be reproduced every time in lentivirally transduced HEK 293T cells (Figure 3.8 and 3.9), probably due to the low viral titers (Figure 4.7 and Table 3.2). One possible explanation is that the number of copies of the transgene integrated into the cell genome was lower than that of transiently transfected cells. Therefore, the minute quantity of free rtTA in the system may not be sufficient to effectively induce strong expression of rtTA and PP2Cβ in the presence of Dox. This is supported by the presence of very faint T7-rtTA bands in V lentivirus-infected cells (Figure 3.8 and 3.11A) and stably established cells (Figure 3.9). RT-qPCR offers greater sensitivity than western blot analysis in gene expression. Due to the difficulties in detecting the gene product via western blot analysis at the protein level, RT-qPCR analysis was employed to identify gene expression at the mRNA level. These set of experiments indicate that Dox treatment of infected HEK 293T cells induced a ~8 fold increase of *rtTA* expression compared to uninduced cells (Figure 3.11B) These results suggest that gene transcription was induced, however, the protein was not produced at levels detectable by immunoblot analyses.

Tet-On systems tend to have a lower Dox sensitivity than that of Tet-Off systems [31], therefore, Dox at a high concentration is required to activate Tet-On systems. Several studies have reported that the Tet-On system requires nearly toxic levels of Dox treatment for controlled induction of transgene expression in animals [28, 85]. Therefore, it remains to be determined whether the concentrations of Dox used in this study were too low to efficiently induce gene expression in lentivirally transduced cells. Previously, researchers have developed variants for rtTA such as rtTA2^{S}-M2^{6} which display significantly 10-100 fold higher sensitivity to Dox treatment compared to WT rtTA [28, 29, 31, 35]. Thus in future studies incorporating more sensitive variants of rtTA could potentially allow more efficient induction whilst using lower Dox concentrations.

It has also been shown that a loss of gene expression in transduced cells could result from extended Dox induction and repeated induction cycles [28, 31]. This could explain why repeated attempts of Dox induction using the same cell failed to show any induction. This could also be a reason for the low induction levels because cells were shown to be efficiently induced by Dox within 72 h post-infection (Figure 3.8). In addition, experimental error could also lead to lower transduction levels in lentivirally infected cells. Lentiviruses were prepared and stored at -80ºC, and an aliquot of lentiviruses stock was thawed prior to infection. This freeze-thaw process could result in a significant reduction of viral titer which could contribute to the loss in transduction efficiency.

4.3 Implications in expected PP2Cβ levels in WT and MUT constructs

PP2Cβ is known to associate with IKKβ, and phosphorylation of IKKβ decreases its kinase activity for the activation of NFκB [1]. An arginine to glycine mutant PP2Cβ (R179G) lacking the ability to phosphorylate IKKβ [1], was used to construct MUT vectors to compare the difference in induction level of PP2Cβ by Dox treatment. Unfortunately, the inducibilty of PP2Cβ could not be determined in the lentivirus-infected HEK 293T cells by western blot. Due to high viral titer of V lentivirus lacking either PP2Cβ WT or MUT (Figure 3.7 and Table 3.2), V lentivirus-infected HEK 293T cells were used in lentiviral infected experiments. The detectable level of rtTA expression in response to Dox was observed from V lentivirus-infected HEK 293T cells, but not shown in a reproducible and consistent manner (Figure 3.8, 3.9 and 3.11A). Because expressed rtTA protein could be unstable in cells, RT-qPCR was used to confirm the inducibility (Figure 3.11B). An eight fold induction in mRNA level between induced and uninduced samples appeared to be low, which implies the difficulty in induction of this novel lentiviral system.

4.4 Summary

Lentiviral vector is commonly used as a gene delivery vehicle that often incorporates a tetracycline inducible system to regulate the transgene expression in target cells, tissues or organs [31, 32, 83]. The transactivator, rtTA, from Tet-On regulatory system was successfully sub-cloned into three lentiviral vectors containing no PP2Cβ control (V),

PP2Cβ wild type (WT) and PP2Cβ mutant (MUT), respectively, which inducibly express rtTA and the transgene in a single plasmid (Figure 3.1 and Figure 3.3). Inducibility of these lentiviral vectors was confirmed by transient transfection in HEK 293T cells. All three vectors revealed responsiveness to Dox in which induced expression of rtTA and PP2Cβ was observed upon 24 h of Dox treatment (Figure 3.5). Lentiviruses were then produced from each lentiviral vector for infection. While induction of rtTA or PP2Cβ expression was observed in lentivirally transduced HEK 293T cells subject to Dox treatment, expression levels were not consistent. Two different strategies were employed to increase the inducibility of this system by: (1) establishing the infected cells at greater cell density followed by Dox induction, and (2) inducing cells within 72 h post-infection. The induction of rtTA was observed in V lentivirus-infected cells, however, this results could not be reproduced consistently (Figure 3.8). Taken together, these results suggest that this novel vector can be used in transient transfection for inducible gene expression; however, its inducibility in transduced cells by infection needs to be improved. In conclusion, this system, which provides inducible gene expression *in vivo*, could be potentially useful for future studies investigating the role of PP2Cβ in regulating signal transduction pathways and molecular ageing.

4.5 Future directions

4.5.1 Addition of tags in vector construct

This study constructed an instantly inducible tetracycline responsive lentiviral vector to express both rtTA and PP2Cβ upon Dox treatment. Unfortunately, the inducibility of lentivirally infected cells was low and very unstable. The infection efficiency was monitored by a standard colony formation assay (Figure 3.7). This method is time consuming due to colony formation of the infected cells under puromycin selection taking up to 3 to 4 weeks. Additionally, the colony staining could not reveal the functional infection quantitatively due to the possible existence of uninfected but resistant cells. Thus, addition of a fluorescent protein marker on the vector, such as green fluorescent protein (GFP), would allow quicker and intuitive visualization of the infection rate by detecting green fluorescence in the cell.

A T7 tag was fused to the N-terminal end of the regulatory activator, rtTA, to allow easy detection of rtTA expression. However, the absence of a tag on PP2Cβ made it difficult to distinguish between Dox induced and endogenous PP2Cβ expression. Since the level of induction turned out to be extremely low by a lentiviral infection in this study, addition of an epitope tag to PP2Cβ would provide a reliable detection of Dox induced PP2Cβ expression and distinguish this between background levels of this protein.

4.5.2 Improvement of lentiviral induction by priming

This Tet-On lentiviral system is activated by the addition of Dox; however, the inducibility of the Dox-dependent system used in this study was low. Dox treatment within three days of post-infection was shown to improve its inducibility (Figure 3.8); however, this improvement was inconspicuous and difficult to detect using immunoblot analysis. It was initially hypothesized that Dox would form a complex with basal levels of free rtTA produced by leaky expression. This Dox-rtTA complex would then associate with TRE to promote downstream transcription of PP2Cβ. Once HEK 293T cells were infected by lentiviruses, PP2Cβ was integrated into each cell genome at a low copy number. Therefore, the background level of rtTA is insufficient to induce expression in the presence of Dox. To examine if this is the case, rtTA could be transiently expressed to see if this priming of rtTA would increase the lentivirus-mediated expression of target genes.

4.5.3 Applications in cellular senescence

In this study, a novel set of lentiviral vectors were designed and constructed which allow delivery of the gene, PP2Cβ, into a variety of cell lines. Using the transformed cell line HEK 293T, we found optimal induction conditions facilitating controlled PP2Cβ expression. Given that lentiviral systems can deliver a gene of interest into non-dividing senescent cells, the next step would be to optimize conditions in other cell lines such as human diploid fibroblasts IMR-90 cells, which are used for *in vitro* ageing studies. The vectors constructed in this study could be useful in future studies to investigate the role of PP2Cβ in a number of pathways such as the senescence (i.e. cellular ageing) pathway mediated by NFκB.

To investigate the role of PP2Cβ in NFκB dependent senescence, different stages of senescent IMR-90 cells would be transduced with inducible PP2Cβ WT and MUT, and allowing comparison of the nuclear expression of altered PP2Cβ. This would be followed by anlysis of protein and RNA levels of the senescence markers (p21, p16, SA-b-Gal, PAI-1) and NFκB responsive genes (ICAM1, TNF, IL6, CCL2, COX2) would be examined to determine whether PP2Cβ expression is corelated with NFκB activity and senescence-associated gene expression. This would test the hypothesis that elevated PP2Cβ expression can block NFκB activated cellular senescence. It would also shed light on whether administration of PP2Cβ as a therapeutic gene in a Dox dependent manner, could have potential in reversing the ageing phenotype even during later stages of senescence.

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Appendices

Appendix I: Primers used for PP2Cβ sequencing.

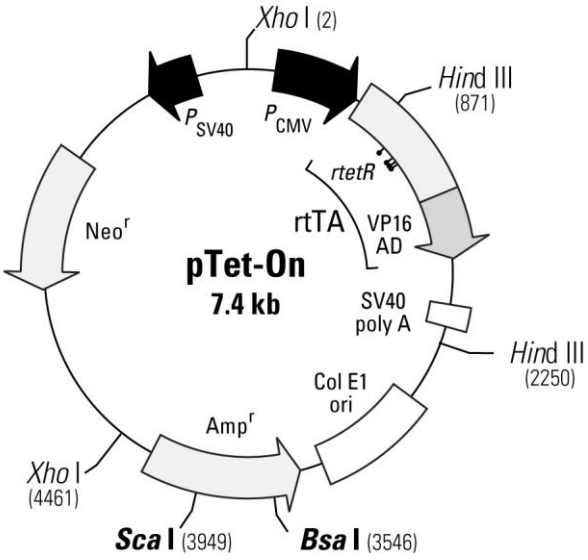
Primer Name	Primer Sequence (5' → 3')*	Appended Sequences
PP2Cβ F	ACTGAGATCTATGTACCCATACGATGTTCCTGACTATGCGGGCATGGGTGCATTTTTGGATAAACCCA	*BglII* site, HA tag
PP2Cβ R	ACTGTCTAGATCATATTTTTTCACCACTCATCTTTGTC	*XbaI* site

*HA tag: blue; restriction sites: red; random end sequences: underlined.

Appendix II: Vector map of pTet-On vector

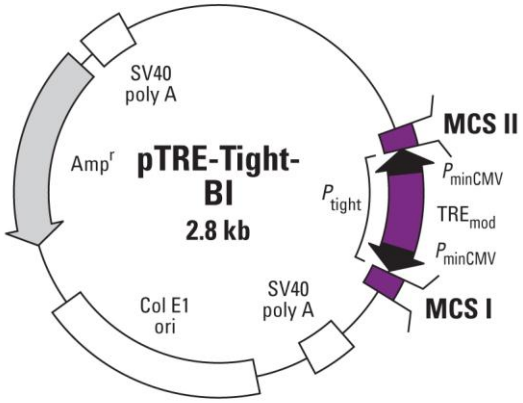
Source: pTet-On® Vector Information from Clontech Laboratories.

http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17935



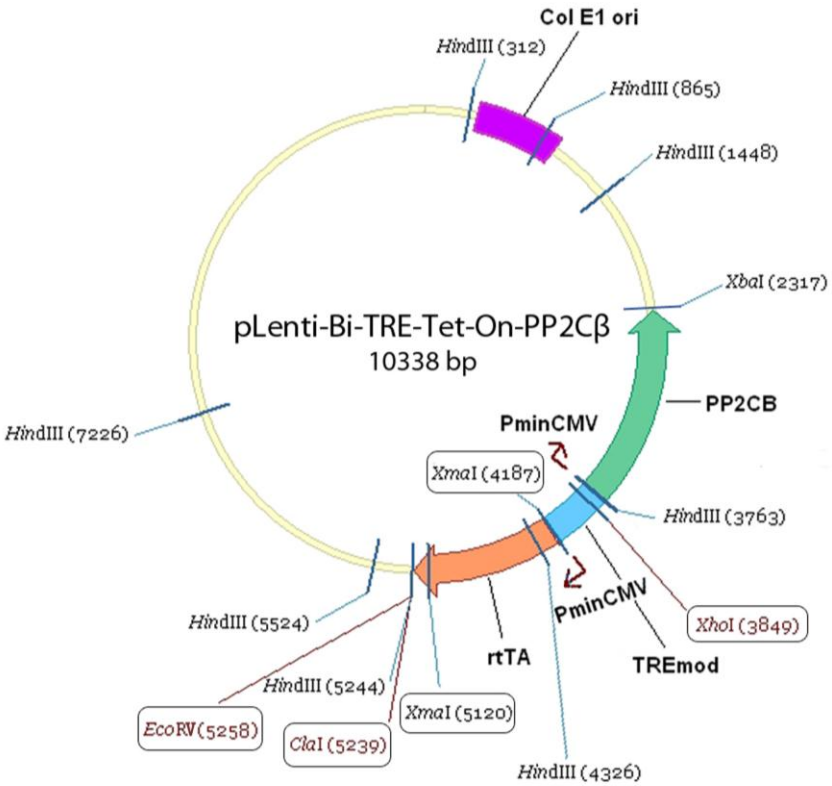
Appendix III: Vector map of pTRE-Tight-BI

Source: pTRE-Tight-BI Vector Information from Clontech Laboratories.
http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17972



Appendix IV: Vector map of pLenti-Bi-Tet-On- PP2Cβ construct

Vector map determined from Invitrogen Vector NTI software.



Appendix V: Sequence of pLenti-Bi-Tet-On-PP2Cβ construct

PP2Cβ indicated in purple, *rtTA* indicated in blue, T7 TAG indicated in light green.

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1       ACGCGTGTAG TCTTATGCAA TACTCTTGTA GTCTTGCAAC ATGGTAACGA
        TGCGCACATC AGAATACGTT ATGAGAACAT CAGAACGTTG TACCATTGCT

51      TGAGTTAGCA ACATGCCTTA CAAGGAGAGA AAAAGCACCG TGCATGCCGA
        ACTCAATCGT TGTACGGAAT GTTCCTCTCT TTTTCGTGGC ACGTACGGCT

101     TTGGTGGAAG TAAGGTGGTA CGATCGTGCC TTATTAGGAA GGCAACAGAC
        AACCACCTTC ATTCCACCAT GCTAGCACGG AATAATCCTT CCGTTGTCTG

151     GGGTCTGACA TGGATTGGAC GAACCACTGA ATTGCCGCAT TGCAGAGATA
        CCCAGACTGT ACCTAACCTG CTTGGTGACT TAACGGCGTA ACGTCTCTAT

201     TTGTATTTAA GTGCCTAGCT CGATACAATA AACGGGTCTC TCTGGTTAGA
        AACATAAATT CACGGATCGA GCTATGTTAT TTGCCCAGAG AGACCAATCT

251     CCAGATCTGA GCCTGGGAGC TCTCTGGCTA ACTAGGGAAC CCACTGCTTA
        GGTCTAGACT CGGACCCTCG AGAGACCGAT TGATCCCTTG GGTGACGAAT

301     AGCCTCAATA AAGCTTGCCT TGAGTGCTTC AAGTAGTGTG TGCCCGTCTG
        TCGGAGTTAT TTCGAACGGA ACTCACGAAG TTCATCACAC ACGGGCAGAC

351     TTGTGTGACT CTGGTAACTA GAGATCCCTC AGACCCTTTT AGTCAGTGTG
        AACACACTGA GACCATTGAT CTCTAGGGAG TCTGGGAAAA TCAGTCACAC

401     GAAAATCTCT AGCAGTGGCG CCCGAACAGG GACCTGAAAG CGAAAGGGAA
        CTTTTAGAGA TCGTCACCGC GGGCTTGTCC CTGGACTTTC GCTTTCCCTT

451     ACCAGAGCTC TCTCGACGCA GGACTCGGCT TGCTGAAGCG CGCACGGCAA
        TGGTCTCGAG AGAGCTGCGT CCTGAGCCGA ACGACTTCGC GCGTGCCGTT

501     GAGGCGAGGG GCGGCGACTG GTGAGTACGC CAAAAATTTT GACTAGCGGA
        CTCCGCTCCC CGCCGCTGAC CACTCATGCG GTTTTTAAAA CTGATCGCCT

551     GGCTAGAAGG AGAGAGATGG GTGCGAGAGC GTCAGTATTA AGCGGGGGAG
        CCGATCTTCC TCTCTCTACC CACGCTCTCG CAGTCATAAT TCGCCCCCTC

601     AATTAGATCG CGATGGGAAA AAATTCGGTT AAGGCCAGGG GGAAAGAAAA
        TTAATCTAGC GCTACCCTTT TTTAAGCCAA TTCCGGTCCC CCTTTCTTTT
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651   AATATAAATT AAAACATATA GTATGGGCAA GCAGGGAGCT AGAACGATTC
      TTATATTTAA TTTTGTATAT CATACCCGTT CGTCCCTCGA TCTTGCTAAG

701   GCAGTTAATC CTGGCCTGTT AGAAACATCA GAAGGCTGTA GACAAATACT
      CGTCAATTAG GACCGGACAA TCTTTGTAGT CTTCCGACAT CTGTTTATGA

751   GGGACAGCTA CAACCATCCC TTCAGACAGG ATCAGAAGAA CTTAGATCAT
      CCCTGTCGAT GTTGGTAGGG AAGTCTGTCC TAGTCTTCTT GAATCTAGTA

801   TATATAATAC AGTAGCAACC CTCTATTGTG TGCATCAAAG GATAGAGATA
      ATATATTATG TCATCGTTGG GAGATAACAC ACGTAGTTTC CTATCTCTAT

851   AAAGACACCA AGGAAGCTTT AGACAAGATA GAGGAAGAGC AAAACAAAAG
      TTTCTGTGGT TCCTTCGAAA TCTGTTCTAT CTCCTTCTCG TTTTGTTTTC

901   TAAGACCACC GCACAGCAAG CGGCCACTGA TCTTCAGACC TGGAGGAGGA
      ATTCTGGTGG CGTGTCGTTC GCCGGTGACT AGAAGTCTGG ACCTCCTCCT

951   GATATGAGGG ACAATTGGAG AAGTGAATTA TATAAATATA AAGTAGTAAA
      CTATACTCCC TGTTAACCTC TTCACTTAAT ATATTTATAT TTCATCATTT

1001  AATTGAACCA TTAGGAGTAG CACCCACCAA GGCAAAGAGA AGAGTGGTGC
      TTAACTTGGT AATCCTCATC GTGGGTGGTT CCGTTTCTCT TCTCACCACG

1051  AGAGAGAAAA AAGAGCAGTG GGAATAGGAG CTTTGTTCCT TGGGTTCTTG
      TCTCTCTTTT TTCTCGTCAC CCTTATCCTC GAAACAAGGA ACCCAAGAAC

1101  GGAGCAGCAG GAAGCACTAT GGGCGCAGCC TCAATGACGC TGACGGTACA
      CCTCGTCGTC CTTCGTGATA CCCGCGTCGG AGTTACTGCG ACTGCCATGT

1151  GGCCAGACAA TTATTGTCTG GTATAGTGCA GCAGCAGAAC AATTTGCTGA
      CCGGTCTGTT AATAACAGAC CATATCACGT CGTCGTCTTG TTAAACGACT

1201  GGGCTATTGA GGCGCAACAG CATCTGTTGC AACTCACAGT CTGGGGCATC
      CCCGATAACT CCGCGTTGTC GTAGACAACG TTGAGTGTCA GACCCCGTAG

1251  AAGCAGCTCC AGGCAAGAAT CCTGGCTGTG GAAAGATACC TAAAGGATCA
      TTCGTCGAGG TCCGTTCTTA GGACCGACAC CTTTCTATGG ATTTCCTAGT

1301  ACAGCTCCTG GGGATTTGGG GTTGCTCTGG AAAACTCATT TGCACCACTG
      TGTCGAGGAC CCCTAAACCC CAACGAGACC TTTTGAGTAA ACGTGGTGAC

1351  CTGTGCCTTG GAATGCTAGT TGGAGTAATA AATCTCTGGA ACAGATTGGA
      GACACGGAAC CTTACGATCA ACCTCATTAT TTAGAGACCT TGTCTAACCT
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1401  ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA TTACACAAGC
      TAGTGTGCTG GACCTACCTC ACCCTGTCTC TTTAATTGTT AATGTGTTCG

1451  TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
      AATTATGTGA GGAATTAACT TCTTAGCGTT TTGGTCGTTC TTTTCTTACT

1501  ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA
      TGTTCTTAAT AACCTTAATC TATTTACCCG TTCAAACACC TTAACCAAAT

1551  ACATAACAAA TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA
      TGTATTGTTT AACCGACACC ATATATTTTA ATAAGTATTA CTATCATCCT

1601  GGCTTGGTAG GTTTAAGAAT AGTTTTTGCT GTACTTTCTA TAGTGAATAG
      CCGAACCATC CAAATTCTTA TCAAAAACGA CATGAAAGAT ATCACTTATC

1651  AGTTAGGCAG GGATATTCAC CATTATCGTT TCAGACCCAC CTCCCAACCC
      TCAATCCGTC CCTATAAGTG GTAATAGCAA AGTCTGGGTG GAGGGTTGGG

1701  CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG TGGAGAGAGA
      GCTCCCCTGG GCTGTCCGGG CTTCCTTATC TTCTTCTTCC ACCTCTCTCT

1751  GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTTAACTTT
      CTGTCTCTGT CTAGGTAAGC TAATCACTTG CCTAGAGCTG CCAATTGAAA

1801  TAAAAGAAAA GGGGGGATTG GGGGGTACAG TGCAGGGGAA AGAATAGTAG
      ATTTTCTTTT CCCCCCTAAC CCCCCATGTC ACGTCCCCTT TCTTATCATC

1851  ACATAATAGC AACAGACATA CAAACTAAAG AATTACAAAA ACAAATTACA
      TGTATTATCG TTGTCTGTAT GTTTGATTTC TTAATGTTTT TGTTTAATGT

1901  AAAATTCAAA ATTTTATCGA AGCAAAAACA GGAAGGCAAA ATGCCGCAAA
      TTTTAAGTTT TAAAATAGCT TCGTTTTTGT CCTTCCGTTT TACGGCGTTT

1951  AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT
      TTTCCCTTAT TCCCGCTGTG CCTTTACAAC TTATGAGTAT GAGAAGGAAA

2001  TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC
      AAGTTATAAT AACTTCGTAA ATAGTCCCAA TAACAGAGTA CTCGCCTATG

2051  ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC CGCGCACATT
      TATAAACTTA CATAAATCTT TTTATTTGTT TATCCCCAAG GCGCGTGTAA

2101  TCCCCGAAAA GTGCCACCTG ACGTCGGCAG TGAAAAAAAT GCTTTATTTG
      AGGGGCTTTT CACGGTGGAC TGCAGCCGTC ACTTTTTTTA CGAAATAAAC
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2151  TGAAATTTGT GATGCTATTG CTTTATTTGT AACCATTATA AGCTGCAATA
      ACTTTAAACA CTACGATAAC GAAATAAACA TTGGTAATAT TCGACGTTAT

2201  AACAAGTTAA CAACAACAAT TGCATTCATT TTATGTTTCA GGTTCAGGGG
      TTGTTCAATT GTTGTTGTTA ACGTAAGTAA AATACAAAGT CCAAGTCCCC

2251  GAGGTGTGGG AGGTTTTTTA AAGCAAGTAA AACCTCTACA AATGTGGTAT
      CTCCACACCC TCCAAAAAAT TTCGTTCATT TTGGAGATGT TTACACCATA
                          XbaI
2301  GGCTGATTAT GATCCTCTAG ATCATATTTT TTCACCACTC ATCTTTGTCC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CCGACTAATA CTAGGAGATC TAGTATAAAA AAGTGGTGAG TAGAAACAGG

2351  CTGCATCTTC ATTAGAGCTG TCTAATTCAG CAAGACCACT TTCTGATTCA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GACGTAGAAG TAATCTCGAC AGATTAAGTC GTTCTGGTGA AAGACTAAGT

2401  GTATGGCTTT CCTGCATTGT CACTGGGTTT CCAGCATCAC TGTTCGAAGA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CATACCGAAA GGACGTAACA GTGACCCAAA GGTCGTAGTG ACAAGCTTCT

2451  AGTAGCTGTG GCAGCTGGTT CAGCAGGGCT TTCTTCTCCC TCTACTTTAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TCATCGACAC CGTCGACCAA GTCGTCCCGA AAGAAGAGGG AGATGAAATC

2501  CTAGCCTGTA ACTAGTCAGC ATCTCCTCCA GAAGTTGTCG GTAGTTTCCC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GATCGGACAT TGATCAGTCG TAGAGGAGGT CTTCAACAGC CATCAAAGGG

2551  CTATGATTAA TTCTCATTTG CCTGAGAGCT TCCACCAATT TTCCCTGTGA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GATACTAATT AAGAGTAAAC GGACTCTCGA AGGTGGTTAA AAGGGACACT

2601  TCCACTTTCC TCTGCTTCAT CGGAGGCCCC ATCACTTTCT CTATGTGGAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AGGTGAAAGG AGACGAAGTA GCCTCCGGGG TAGTGAAAGA GATACACCTA

2651  TCAGTCTACT ATAAACAGCT TCAATAACAT TACGCTTGCC AGCAAGACCT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AGTCAGATGA TATTTGTCGA AGTTATTGTA ATGCGAACGG TCGTTCTGGA

2701  CCCCCAGGAG GCAAATTTGG GATATTTTCT GCAGACAAGA TGCGCATGAC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GGGGGTCCTC CGTTTAAACC CTATAAAAGA CGTCTGTTCT ACGCGTACTG
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2751  ATGGGCAAGA TCAGGCATTC CTTCCTCGCC AGACTTCTCC ATAATCTCTT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TACCCGTTCT AGTCCGTAAG GAAGGAGCGG TCTGAAGAGG TATTAGAGAA

2801  CAACCCGTGA TTCCAAGTGC TTATCCAACT CTGAATCTTT TTTCACCGCT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GTTGGGCACT AAGGTTCACG AATAGGTTGA GACTTAGAAA AAAGTGGCGA

2851  TCATCTGAGA CCTTGGGAGC ATTTGAAAAG CAAACTAGTA CAATACTCAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AGTAGACTCT GGAACCCTCG TAAACTTTTC GTTTGATCAT GTTATGAGTA

2901  GTTATCTCGA CTTCCCTTGT GTAAACAAGT GTCCACTACC CAATTGCACA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CAATAGAGCT GAAGGGAACA CATTTGTTCA CAGGTGATGG GTTAACGTGT

2951  CATTTTCCAG GTCATCAGAT ACCTCAAGCC TAGATTTAAC ATATTCACAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GTAAAAGGTC CAGTAGTCTA TGGAGTTCGG ATCTAAATTG TATAAGTGTC

3001  AGCTCCTCAT TACTCATAAC ATCCCAGATC CCATCACAAG CCAAGATGAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TCGAGGAGTA ATGAGTATTG TAGGGTCTAG GGTAGTGTTC GGTTCTACTA

3051  AAATTCATCC TCTTCTGCTC TTAAAATTTC ATAAACCTCA GGCTCTGGAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTTAAGTAGG AGAAGACGAG AATTTTAAAG TATTTGGAGT CCGAGACCTC

3101  AAACAAGTTG TTCTGTTGGG CCCTTGCCAT CAACACACTT GTAATCATAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTTGTTCAAC AAGACAACCC GGGAACGGTA GTTGTGTGAA CATTAGTATC

3151  TCCCCCAGAG CACGAGATAC TGCTAATGAA CCATTAACAC GTTGTATCAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AGGGGGTCTC GTGCTCTATG ACGATTACTT GGTAATTGTG CAACATAGTA

3201  CACGCTGCCT CCTGCATTTT GGATTCGCTC CTTTTCCCTT GGATTGCAAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GTGCGACGGA GGACGTAAAA CCTAAGCGAG GAAAAGGGAA CCTAACGTTC
                                G R179G[1]
3251  GTTTGTGATC CTGGGTAGAA AAGCAGACTT GTCCATTCCT ATACAGAACA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CAAACACTAG GACCCATCTT TTCGTCTGAA CAGGTAAGGA TATGTCTTGT
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[1] Refer to Prajapati *et al.* [1].

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3301  GCACGTGAAT CACCACAGTT GATAAAGTAG ATATGCTTAG GTGAAATCAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CGTGCACTTA GTGGTGTCAA CTATTTCATC TATACGAATC CACTTTAGTA

3351  AACTCCCACT GCAGTTGAAC CACTCCTGTC CATCCCGTTT CTGAGGTCTG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTGAGGGTGA CGTCAACTTG GTGAGGACAG GTAGGGCAAA GACTCCAGAC

3401  AAAAGTTACG CATGTATTCA TCAATTTTCA AAAATCCAGT TCTGATACCA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTTTCAATGC GTACATAAGT AGTTAAAAGT TTTTAGGTCA AGACTATGGT

3451  TTCTTAACAT TTTCCACTGA AAGCTCAAGA GCAGATCCTG ATTTTCCAGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AAGAATTGTA AAAGGTGACT TTCGAGTTCT CGTCTAGGAC TAAAAGGTCG

3501  TGCCCTAAAG TCTTCGTTAG TAGTGATGTG TTCTAATAAA TGTGTTGAGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      ACGGGATTTC AGAAGCAATC ATCACTACAC AAGATTATTT ACACAACTCG

3551  AGTAATTTGC CACTCGGGAT CCAGCATGAC CATCATAAAC TGCAAAAAAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TCATTAAACG GTGAGCCCTA GGTCGTACTG GTAGTATTTG ACGTTTTTTA

3601  GACCAGTCTT CCAAGCCGTG AGGAATACCT ACAACAGCTG TGTGTGCATC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CTGGTCAGAA GGTTCGGCAC TCCTTATGGA TGTTGTCGAC ACACACGTAG

3651  TTCCATTTCC ACTCTCCATC CTTGCATGCT GCTCAGGCCA TAACGTAAAC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AAGGTAAAGG TGAGAGGTAG GAACGTACGA CGAGTCCGGT ATTGCATTTG

3701  CATTCCCAGC ACCATGAGCA TTATGTTTTT CAGTTTTGGG TTTATCCAAA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GTAAGGGTCG TGGTACTCGT AATACAAAAA GTCAAAACCC AAATAGGTTT
                  HindIII
3751  AATGCACCCA TAAGCTTGAA TTCTCCAGGC GATCTGACGG TTCACTAAAC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTACGTGGGT ATTCGAACTT AAGAGGTCCG CTAGACTGCC AAGTGATTTG
      <-PP2Cβ                                          XhoI
3801  GAGCTCTGCT TATATAGGCC TCCCACCGTA CACGCCACCT CGACATACTC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CTCGAGACGA ATATATCCGG AGGGTGGCAT GTGCGGTGGA GCTGTATGAG
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3851  GAGTTTACTC CCTATCAGTG ATAGAGAACG TATGTCGAGT TTACTCCCTA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CTCAAATGAG GGATAGTCAC TATCTCTTGC ATACAGCTCA AATGAGGGAT

3901  TCAGTGATAG AGAACGATGT CGAGTTTACT CCCTATCAGT GATAGAGAAC
      AGTCACTATC TCTTGCTACA GCTCAAATGA GGGATAGTCA CTATCTCTTG

3951  GTATGTCGAG TTTACTCCCT ATCAGTGATA GAGAACGTAT GTCGAGTTTA
      CATACAGCTC AAATGAGGGA TAGTCACTAT CTCTTGCATA CAGCTCAAAT

4001  CTCCCTATCA GTGATAGAGA ACGTATGTCG AGTTTATCCC TATCAGTGAT
      GAGGGATAGT CACTATCTCT TGCATACAGC TCAAATAGGG ATAGTCACTA

4051  AGAGAACGTA TGTCGAGTTT ACTCCCTATC AGTGATAGAG AACGTATGTC
      TCTCTTGCAT ACAGCTCAAA TGAGGGATAG TCACTATCTC TTGCATACAG

4101  GAGGTAGGCG TGTACGGTGG GAGGCCTATA TAAGCAGAGC TCGTTTAGTG
      CTCCATCCGC ACATGCCACC CTCCGGATAT ATTCGTCTCG AGCAAATCAC
                                                XmaI
4151  AACCGTCAGA TCGCCTGGAG ATTTCGAGCT CGGTACCCGG GCACCATGGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTGGCAGTCT AGCGGACCTC TAAAGCTCGA GCCATGGGCC CGTGGTACCG
                T7 Tag                    rtTA  XbaI
4201  TAGCATGACT GGTGGACAGC AAATGGGTAT GTCTAGATTA GATAAAAGTA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      ATCGTACTGA CCACCTGTCG TTTACCCATA CAGATCTAAT CTATTTTCAT

4251  AAGTGATTAA CAGCGCATTA GAGCTGCTTA ATGAGGTCGG AATCGAAGGT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTCACTAATT GTCGCGTAAT CTCGACGAAT TACTCCAGCC TTAGCTTCCA
                                  HindIII
4301  TTAACAACCC GTAAACTCGC CCAGAAGCTT GGTGTAGAGC AGCCTACACT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AATTGTTGGG CATTTGAGCG GGTCTTCGAA CCACATCTCG TCGGATGTGA

4351  GTATTGGCAT GTAAAAAATA AGCGGGCTTT GCTCGACGCC TTAGCCATTG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CATAACCGTA CATTTTTTAT TCGCCCGAAA CGAGCTGCGG AATCGGTAAC

4401  AGATGTTAGA TAGGCACCAT ACTCACTTTT GCCCTTTAAA AGGGGAAAGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TCTACAATCT ATCCGTGGTA TGAGTGAAAA CGGGAAATTT TCCCCTTTCG
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4451  TGGCAAGATT TTTTACGCAA TAACGCTAAA AGTTTTAGAT GTGCTTTACT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      ACCGTTCTAA AAAATGCGTT ATTGCGATTT TCAAAATCTA CACGAAATGA

4501  AAGTCATCGC AATGGAGCAA AAGTACATTC AGATACACGG CCTACAGAAA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTCAGTAGCG TTACCTCGTT TTCATGTAAG TCTATGTGCC GGATGTCTTT

4551  AACAGTATGA AACTCTCGAA AATCAATTAG CCTTTTTATG CCAACAAGGT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TTGTCATACT TTGAGAGCTT TTAGTTAATC GGAAAAATAC GGTTGTTCCA

4601  TTTTCACTAG AGAACGCGTT ATATGCACTC AGCGCTGTGG GGCATTTTAC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AAAAGTGATC TCTTGCGCAA TATACGTGAG TCGCGACACC CCGTAAAATG

4651  TTTAGGTTGC GTATTGGAAG ATCAAGAGCA TCAAGTCGCT AAAGAAGAAA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AAATCCAACG CATAACCTTC TAGTTCTCGT AGTTCAGCGA TTTCTTCTTT

4701  GGGAAACACC TACTACTGAT AGTATGCCGC CATTATTACG ACAAGCTATC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CCCTTTGTGG ATGATGACTA TCATACGGCG GTAATAATGC TGTTCGATAG

4751  GAATTATTTG ATCACCAAGG TGCAGAGCCA GCCTTCTTAT TCGGCCTTGA
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CTTAATAAAC TAGTGGTTCC ACGTCTCGGT CGGAAGAATA AGCCGGAACT

4801  ATTGATCATA TGCGGATTAG AAAAACAACT TAAATGTGAA AGTGGGTCCG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TAACTAGTAT ACGCCTAATC TTTTTGTTGA ATTTACACTT TCACCCAGGC

4851  CGTACAGCCG CGCGCGTACG AAAAACAATT ACGGGTCTAC CATCGAGGGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GCATGTCGGC GCGCGCATGC TTTTTGTTAA TGCCCAGATG GTAGCTCCCG

4901  CTGCTCGATC TCCCGGACGA CGACGCCCCC GAAGAGGCGG GGCTGGCGGC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GACGAGCTAG AGGGCCTGCT GCTGCGGGGG CTTCTCCGCC CCGACCGCCG

4951  TCCGCGCCTG TCCTTTCTCC CCGCGGGACA CACGCGCAGA CTGTCGACGG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      AGGCGCGGAC AGGAAAGAGG GGCGCCCTGT GTGCGCGTCT GACAGCTGCC
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5001  CCCCCCCGAC CGATGTCAGC CTGGGGGACG AGCTCCACTT AGACGGCGAG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GGGGGGGCTG GCTACAGTCG GACCCCCTGC TCGAGGTGAA TCTGCCGCTC

5051  GACGTGGCGA TGGCGCATGC CGACGCGCTA GACGATTTCG ATCTGGACAT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CTGCACCGCT ACCGCGTACG GCTGCGCGAT CTGCTAAAGC TAGACCTGTA
                                XmaI
5101  GTTGGGGGAC GGGGATTCCC CGGGTCCGGG ATTTACCCCC CACGACTCCG
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      CAACCCCCTG CCCCTAAGGG GCCCAGGCCC TAAATGGGGG GTGCTGAGGC

5151  CCCCCTACGG CGCTCTGGAT ATGGCCGACT TCGAGTTTGA GCAGATGTTT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      GGGGGATGCC GCGAGACCTA TACCGGCTGA AGCTCAAACT CGTCTACAAA
                                                    ClaI
5201  ACCGATGCCC TTGGAATTGA CGAGTACGGT GGGTAGATCG ATAAGCTTGT
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
      TGGCTACGGG AACCTTAACT GCTCATGCCA CCCATCTAGC TATTCGAACA
          EcoRI
5251  CGACGATATC TCCAGATTTG GCCGCAAGGA TCTGCGATCG CTCCGGTGCC
      |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
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5301  CGTCAGTGGG CAGAGCGCAC ATCGCCCACA GTCCCCGAGA AGTTGGGGGG
      GCAGTCACCC GTCTCGCGTG TAGCGGGTGT CAGGGGCTCT TCAACCCCCC

5351  AGGGGTCGGC AATTGAACGG GTGCCTAGAG AAGGTGGCGC GGGGTAAACT
      TCCCCAGCCG TTAACTTGCC CACGGATCTC TTCCACCGCG CCCCATTTGA

5401  GGGAAAGTGA TGTCGTGTAC TGGCTCCGCC TTTTTCCCGA GGGTGGGGGA
      CCCTTTCACT ACAGCACATG ACCGAGGCGG AAAAAGGGCT CCCACCCCCT

5451  GAACCGTATA TAAGTGCAGT AGTCGCCGTG AACGTTCTTT TTCGCAACGG
      CTTGGCATAT ATTCACGTCA TCAGCGGCAC TTGCAAGAAA AAGCGTTGCC

5501  GTTTGCCGCC AGAACACAGC TGAAGCTTCG AGGGGCTCGC ATCTCTCCTT
      CAAACGGCGG TCTTGTGTCG ACTTCGAAGC TCCCCGAGCG TAGAGAGGAA

5551  CACGCGCCCG CCGCCCTACC TGAGGCCGCC ATCCACGCCG GTTGAGTCGC
      GTGCGCGGGC GGCGGGATGG ACTCCGGCGG TAGGTGCGGC CAACTCAGCG

5601  GTTCTGCCGC CTCCCGCCTG TGGTGCCTCC TGAACTGCGT CCGCCGTCTA
      CAAGACGGCG GAGGGCGGAC ACCACGGAGG ACTTGACGCA GGCGGCAGAT
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5651  GGTAAGTTTA AAGCTCAGGT CGAGACCGGG CCTTTGTCCG GCGCTCCCTT
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5701  GGAGCCTACC TAGACTCAGC CGGCTCTCCA CGCTTTGCCT GACCCTGCTT
      CCTCGGATGG ATCTGAGTCG GCCGAGAGGT GCGAAACGGA CTGGGACGAA

5751  GCTCAACTCT ACGTCTTTGT TTCGTTTTCT GTTCTGCGCC GTTACAGATC
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5801  CAAGCTGTGA CCGGCGCCTA CGCTAGATGA CCGAGTACAA GCCCACGGTG
      GTTCGACACT GGCCGCGGAT GCGATCTACT GGCTCATGTT CGGGTGCCAC

5851  CGCCTCGCCA CCCGCGACGA CGTCCCCAGG GCCGTACGCA CCCTCGCCGC
      GCGGAGCGGT GGGCGCTGCT GCAGGGGTCC CGGCATGCGT GGGAGCGGCG

5901  CGCGTTCGCC GACTACCCCG CCACGCGCCA CACCGTCGAT CCGGACCGCC
      GCGCAAGCGG CTGATGGGGC GGTGCGCGGT GTGGCAGCTA GGCCTGGCGG

5951  ACATCGAGCG GGTCACCGAG CTGCAAGAAC TCTTCCTCAC GCGCGTCGGG
      TGTAGCTCGC CCAGTGGCTC GACGTTCTTG AGAAGGAGTG CGCGCAGCCC

6001  CTCGACATCG GCAAGGTGTG GGTCGCGGAC GACGGCGCCG CGGTGGCGGT
      GAGCTGTAGC CGTTCCACAC CCAGCGCCTG CTGCCGCGGC GCCACCGCCA

6051  CTGGACCACG CCGGAGAGCG TCGAAGCGGG GGCGGTGTTC GCCGAGATCG
      GACCTGGTGC GGCCTCTCGC AGCTTCGCCC CCGCCACAAG CGGCTCTAGC

6101  GCCCGCGCAT GGCCGAGTTG AGCGGTTCCC GGCTGGCCGC GCAGCAACAG
      CGGGCGCGTA CCGGCTCAAC TCGCCAAGGG CCGACCGGCG CGTCGTTGTC

6151  ATGGAAGGCC TCCTGGCGCC GCACCGGCCC AAGGAGCCCG CGTGGTTCCT
      TACCTTCCGG AGGACCGCGG CGTGGCCGGG TTCCTCGGGC GCACCAAGGA

6201  GGCCACCGTC GGCGTCTCGC CCGACCACCA GGGCAAGGGT CTGGGCAGCG
      CCGGTGGCAG CCGCAGAGCG GGCTGGTGGT CCCGTTCCCA GACCCGTCGC

6251  CCGTCGTGCT CCCCGGAGTG GAGGCGGCCG AGCGCGCCGG GGTGCCCGCC
      GGCAGCACGA GGGGCCTCAC CTCCGCCGGC TCGCGCGGCC CCACGGGCGG

6301  TTCCTGGAGA CCTCCGCGCC CCGCAACCTC CCCTTCTACG AGCGGCTCGG
      AAGGACCTCT GGAGGCGCGG GGCGTTGGAG GGGAAGATGC TCGCCGAGCC

6351  CTTCACCGTC ACCGCCGACG TCGAGGTGCC CGAAGGACCG CGCACCTGGT
      GAAGTGGCAG TGGCGGCTGC AGCTCCACGG GCTTCCTGGC GCGTGGACCA
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6401  GCATGACCCG CAAGCCCGGT GCCTGAGTCG ACAATCAACC TCTGGATTAC
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6451  AAAATTTGTG AAAGATTGAC TGGTATTCTT AACTATGTTG CTCCTTTTAC
      TTTTAAACAC TTTCTAACTG ACCATAAGAA TTGATACAAC GAGGAAAATG

6501  GCTATGTGGA TACGCTGCTT TAATGCCTTT GTATCATGCT ATTGCTTCCC
      CGATACACCT ATGCGACGAA ATTACGGAAA CATAGTACGA TAACGAAGGG

6551  GTATGGCTTT CATTTTCTCC TCCTTGTATA AATCCTGGTT GCTGTCTCTT
      CATACCGAAA GTAAAAGAGG AGGAACATAT TTAGGACCAA CGACAGAGAA

6601  TATGAGGAGT TGTGGCCCGT TGTCAGGCAA CGTGGCGTGG TGTGCACTGT
      ATACTCCTCA ACACCGGGCA ACAGTCCGTT GCACCGCACC ACACGTGACA

6651  GTTTGCTGAC GCAACCCCCA CTGGTTGGGG CATTGCCACC ACCTGTCAGC
      CAAACGACTG CGTTGGGGGT GACCAACCCC GTAACGGTGG TGGACAGTCG

6701  TCCTTTCCGG GACTTTCGCT TTCCCCCTCC CTATTGCCAC GGCGGAACTC
      AGGAAAGGCC CTGAAAGCGA AAGGGGGAGG GATAACGGTG CCGCCTTGAG

6751  ATCGCCGCCT GCCTTGCCCG CTGCTGGACA GGGGCTCGGC TGTTGGGCAC
      TAGCGGCGGA CGGAACGGGC GACGACCTGT CCCCGAGCCG ACAACCCGTG

6801  TGACAATTCC GTGGTGTTGT CGGGGAAATC ATCGTCCTTT CCTTGGCTGC
      ACTGTTAAGG CACCACAACA GCCCCTTTAG TAGCAGGAAA GGAACCGACG

6851  TCGCCTGTGT TGCCACCTGG ATTCTGCGCG GGACGTCCTT CTGCTACGTC
      AGCGGACACA ACGGTGGACC TAAGACGCGC CCTGCAGGAA GACGATGCAG

6901  CCTTCGGCCC TCAATCCAGC GGACCTTCCT TCCCGCGGCC TGCTGCCGGC
      GGAAGCCGGG AGTTAGGTCG CCTGGAAGGA AGGGCGCCGG ACGACGGCCG

6951  TCTGCGGCCT CTTCCGCGTC TTCGCCTTCG CCCTCAGACG AGTCGGATCT
      AGACGCCGGA GAAGGCGCAG AAGCGGAAGC GGGAGTCTGC TCAGCCTAGA

7001  CCCTTTGGGC CGCCTCCCCG CCTGGTACCT TTAAGACCAA TGACTTACAA
      GGGAAACCCG GCGGAGGGGC GGACCATGGA AATTCTGGTT ACTGAATGTT

7051  GGCAGCTGTA GATCTTAGCC ACTTTTTAAA AGAAAAGGGG GGACTGGAAG
      CCGTCGACAT CTAGAATCGG TGAAAAATTT TCTTTTCCCC CCTGACCTTC

7101  GGCTAATTCA CTCCCAACGA AAATAAGATC TGCTTTTTGC TTGTACTGGG
      CCGATTAAGT GAGGGTTGCT TTTATTCTAG ACGAAAAACG AACATGACCC
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7151  TCTCTCTGGT TAGACCAGAT CTGAGCCTGG GAGCTCTCTG GCTAACTAGG
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                                 HindIII
7201  GAACCCACTG CTTAAGCCTC AATAAAGCTT GCCTTGAGTG CTTCAAGTAG
      CTTGGGTGAC GAATTCGGAG TTATTTCGAA CGGAACTCAC GAAGTTCATC

7251  TGTGTGCCCG TCTGTTGTGT GACTCTGGTA ACTAGAGATC CCTCAGACCC
      ACACACGGGC AGACAACACA CTGAGACCAT TGATCTCTAG GGAGTCTGGG

7301  TTTTAGTCAG TGTGGAAAAT CTCTAGCAGT AGTAGTTCAT GTCATCTTAT
      AAAATCAGTC ACACCTTTTA GAGATCGTCA TCATCAAGTA CAGTAGAATA

7351  TATTCAGTAT TTATAACTTG CAAAGAAATG AATATCAGAG AGTGAGAGGA
      ATAAGTCATA AATATTGAAC GTTTCTTTAC TTATAGTCTC TCACTCTCCT

7401  ACTTGTTTAT TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA
      TGAACAAATA ACGTCGAATA TTACCAATGT TTATTTCGTT ATCGTAGTGT

7451  AATTTCACAA ATAAAGCATT TTTTTCACTG CATTCTAGTT GTGGTTTGTC
      TTAAAGTGTT TATTTCGTAA AAAAAGTGAC GTAAGATCAA CACCAAACAG

7501  CAAACTCATC AATGTATCTT ATCATGTCTG GCTCTAGCTA TCCCGCCCCT
      GTTTGAGTAG TTACATAGAA TAGTACAGAC CGAGATCGAT AGGGCGGGGA

7551  AACTCCGCCC AGTTCCGCCC ATTCTCCGCC CCATGGCTGA CTAATTTTTT
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7601  TTATTTATGC AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG
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7651  TAGTGAGGAG GCTTTTTTGG AGGCCTAGAC TTTTGCAGAG ACGGCCCAAA
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7701  TTCGTAATCA TGGTCATAGC TGTTTCCTGT GTGAAATTGT TATCCGCTCA
      AAGCATTAGT ACCAGTATCG ACAAAGGACA CACTTTAACA ATAGGCGAGT

7751  CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGTGTAA AGCCTGGGGT
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7801  GCCTAATGAG TGAGCTAACT CACATTAATT GCGTTGCGCT CACTGCCCGC
      CGGATTACTC ACTCGATTGA GTGTAATTAA CGCAACGCGA GTGACGGGCG

7851  TTTCCAGTCG GGAAACCTGT CGTGCCAGCT GCATTAATGA ATCGGCCAAC
      AAAGGTCAGC CCTTTGGACA GCACGGTCGA CGTAATTACT TAGCCGGTTG
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7901  GCGCGGGGAG AGGCGGTTTG CGTATTGGGC GCTCTTCCGC TTCCTCGCTC
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7951  ACTGACTCGC TGCGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA
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8001  CTCAAAGGCG GTAATACGGT TATCCACAGA ATCAGGGGAT AACGCAGGAA
      GAGTTTCCGC CATTATGCCA ATAGGTGTCT TAGTCCCCTA TTGCGTCCTT

8051  AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC
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8101  GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACAA
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8151  AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT
      TTTAGCTGCG AGTTCAGTCT CCACCGCTTT GGGCTGTCCT GATATTTCTA

8201  ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC
      TGGTCCGCAA AGGGGGACCT TCGAGGGAGC ACGCGAGAGG ACAAGGCTGG

8251  CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG GAAGCGTGGc
      GACGGCGAAT GGCCTATGGA CAGGCGGAAA GAGGGAAGCC CTTCGCACCG

8301  GCTTTCTCAT AGCTCACGCT GTAGGTATCT CAGTTCGGTG TAGGTCGTTC
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8351  GCTCCAAGCT GGGCTGTGTG CACGAACCCC CCGTTCAGCC CGACCGCTGC
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8401  GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA GACACGACTT
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8451  ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG
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8501  TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT
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8551  AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG TTACCTTCGG
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8601  AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCACC GCTGGTAGCG
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8651  GTGGTTTTTT TGTTTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT
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8701  CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGAACGA
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8751  AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA
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8801  CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT CTAAAGTATA
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8851  TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA GTGAGGCACC
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8901  TATCTCAGCG ATCTGTCTAT TTCGTTCATC CATAGTTGCC TGACTCCCCG
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8951  TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT
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9001  GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT
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9051  AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT GCAACTTTAT
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9101  CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG AGTAAGTAGT
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9151  TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTA CAGGCATCGT
      AGCGGTCAAT TATCAAACGC GTTGCAACAA CGGTAACGAT GTCCGTAGCA

9201  GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC GGTTCCCAAC
      CCACAGTGCG AGCAGCAAAC CATACCGAAG TAAGTCGAGG CCAAGGGTTG

9251  GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA AGCGGTTAGC
      CTAGTTCCGC TCAATGTACT AGGGGGTACA ACACGTTTTT TCGCCAATCG

9301  TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG CAGTGTTATC
      AGGAAGCCAG GAGGCTAGCA ACAGTCTTCA TTCAACCGGC GTCACAATAG

9351  ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC ATGCCATCCG
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9401   TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC ATTCTGAGAA
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9451   TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA
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9501   TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT GGAAAACGTT
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9551   CTTCGGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTCG
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9601   ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT TTACTTTCAC
       TACATTGGGT GAGCACGTGG GTTGACTAGA AGTCGTAGAA AATGAAAGTG

9651   CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC GCAAAAAAGG
       GTCGCAAAGA CCCACTCGTT TTTGTCCTTC CGTTTTACGG CGTTTTTTCC

9701   GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT CCTTTTTCAA
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9751   TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT
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9801   TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC
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9851   GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC
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9901   TATAAAAATA GGCGTATCAC GAGGCCCTTT CGTCTCGCGC GTTTCGGTGA
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9951   TGACGGTGAA AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT
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10001  GTCTGTAAGC GGATGCCGGG AGCAGACAAG CCCGTCAGGG CGCGTCAGCG
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10051  GGTGTTGGCG GGTGTCGGGG CTGGCTTAAC TATGCGGCAT CAGAGCAGAT
       CCACAACCGC CCACAGCCCC GACCGAATTG ATACGCCGTA GTCTCGTCTA

10101  TGTACTGAGA GTGCACCATA TGCGGTGTGA AATACCGCAC AGATGCGTAA
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10151 GGAGAAAATA CCGCATCAGG CGCCATTCGC CATTCAGGCT GCGCAACTGT
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10201 TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA
      ACCCTTCCCG CTAGCCACGC CCGGAGAAGC GATAATGCGG TCGACCGCTT

10251 AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC
      TCCCCCTACA CGACGTTCCG CTAATTCAAC CCATTGCGGT CCCAAAAGGG

10301 AGTCACGACG TTGTAAAACG ACGGCCAGTG CCAAGCTG
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Appendix VI: Standard curves of *rtTA* and *GUSB* for statistical analysis.

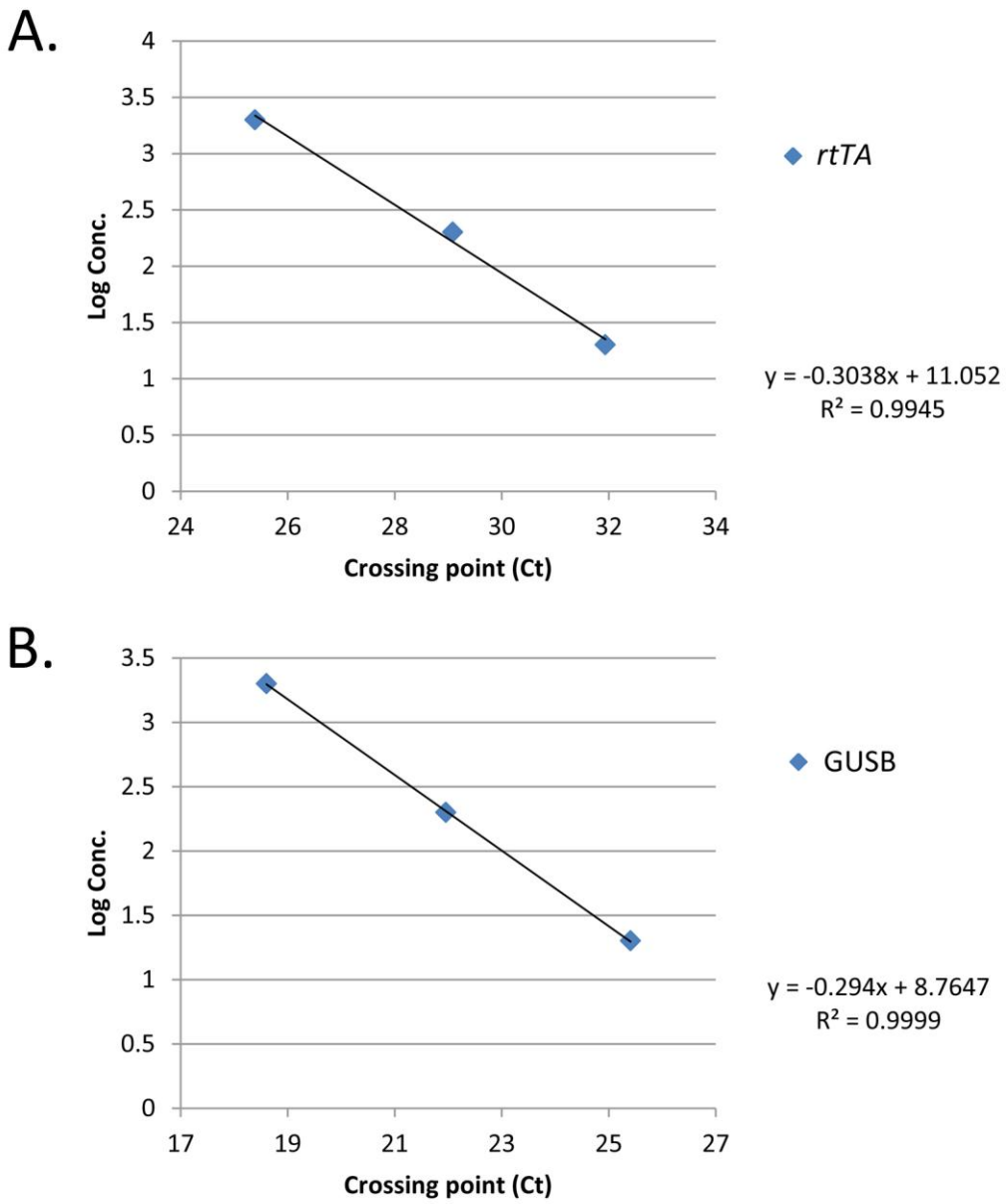

Figure A.VI: Standard curves of *rtTA* and *GUSB* constructed for statistical analysis. Log concentration of 2000, 200 and 20 was used for construction, corresponding to 1:5, 1:50 and 1:500 serial dilutions of each sample. (A), (B) are standard curves for *rtTA* and GUSB, respectively.

Appendix VII: Amplification curves, standard curve and melting peaks for *rtTA*.

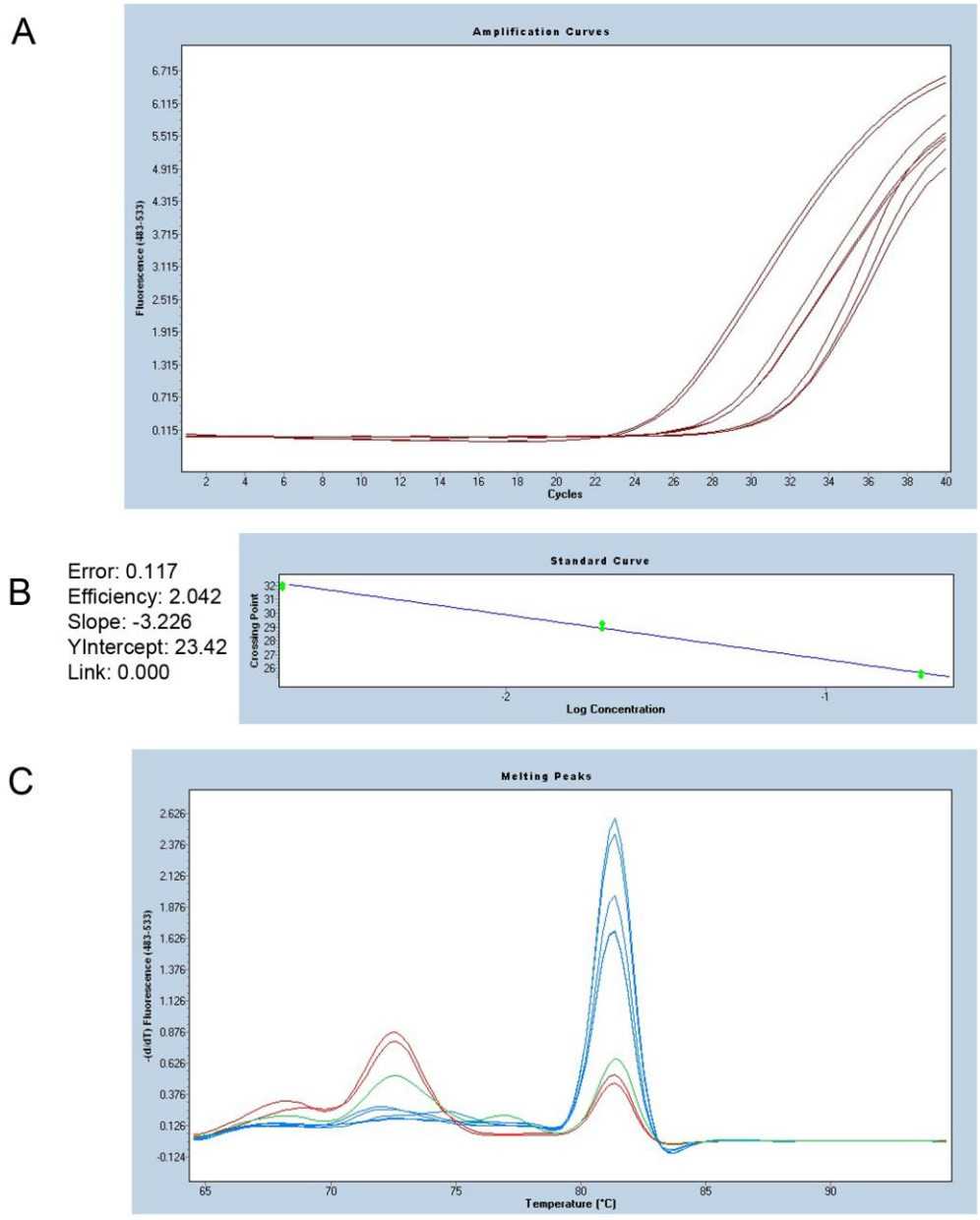

Figure A.VII: Standard curve optimization for *rtTA* amplicon using quantitative real-time PCR. (A) Amplification curves of a 10 fold serial dilution were used for construction of a standard curve. (B) Corresponding standard curve with an amplification efficiency of 2.042. (C) Corresponding melting peaks indicate specificity of PCR.

Appendix VIII: Amplification curves, standard curve and melting peaks for *GUSB*.

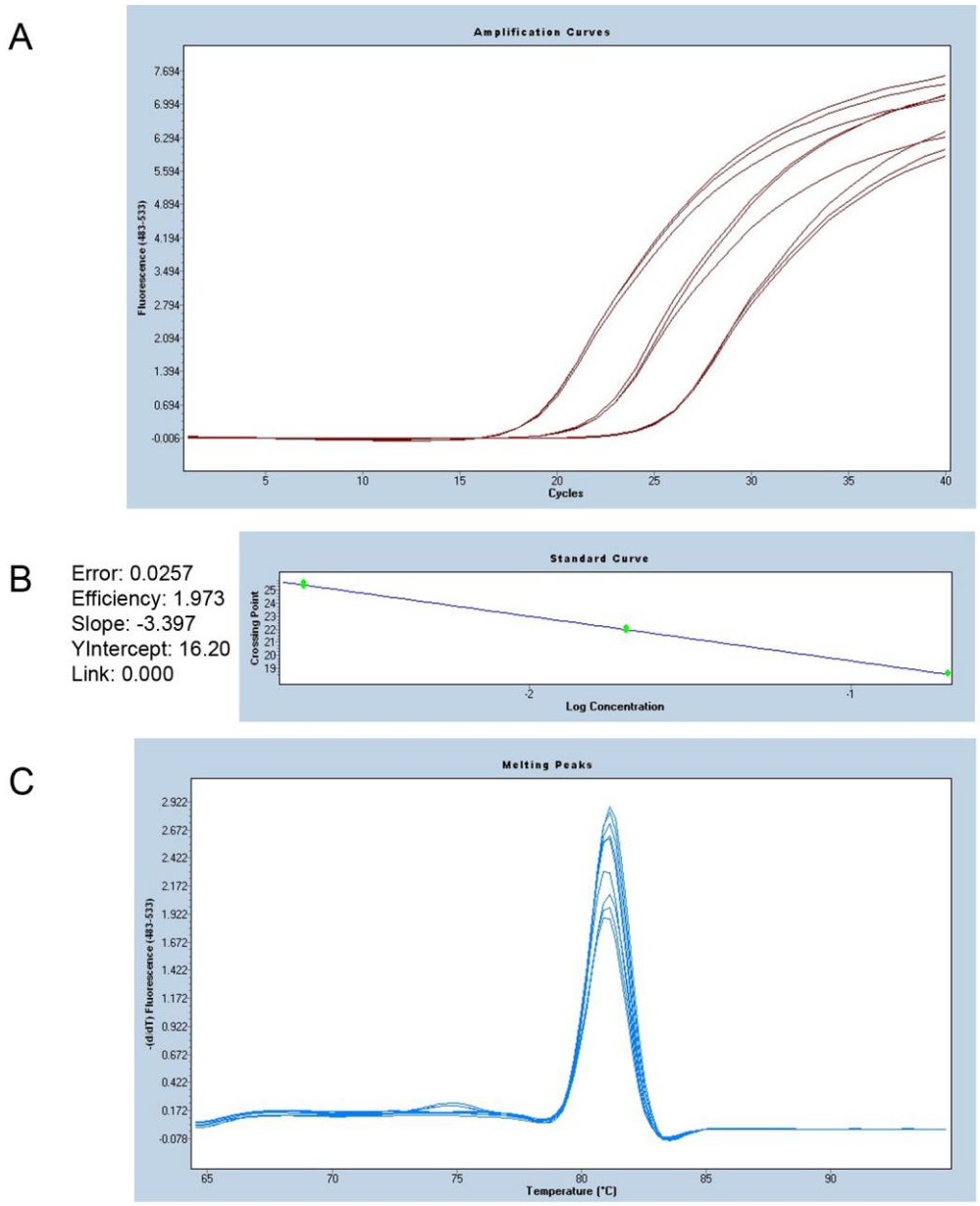

Figure A.VIII: Standard curve optimization for *GUSB* amplicon using quantitative real-time PCR. (A) Amplification curves of a 10 fold serial dilution were used for construction of a standard curve. (B) Corresponding standard curve with an amplification efficiency of 1.97. (C) Corresponding melting peaks indicate specificity of PCR.