

All-in-one Tet-On Inducible Vectors



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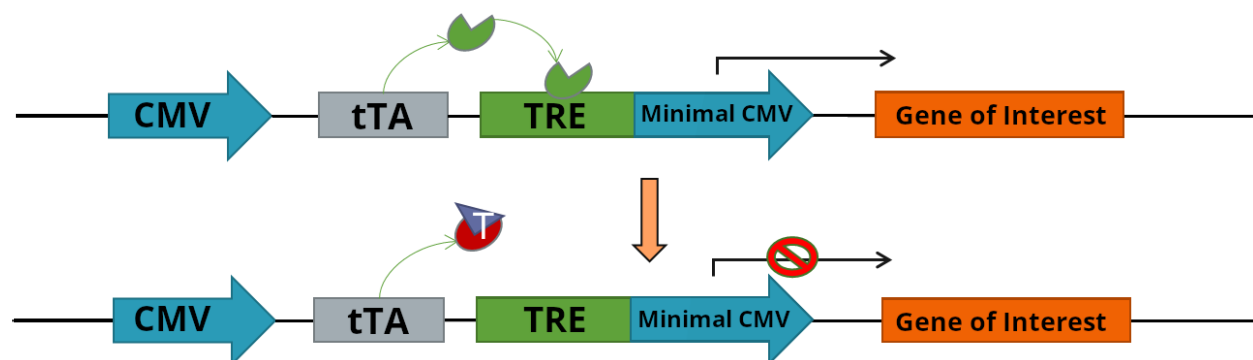
Introduction

The tetracycline inducible system constitutes of two components, a tetracycline dependent promoter and a tetracycline-controlled regulator.

A tetracycline-dependent promoter is developed by placing a tetracycline response element (TRE) upstream of a minimal promoter. A TRE is a series of 7 repeats of a 19 nucleotide tetracycline operator (tetO). It can be recognized by the tetracycline repressor (tetR) in the endogenous bacterial system, which then inhibits the transcription of downstream gene. In the presence of tetracycline, tetR will bind to tetracycline and not to TRE. The transcription of the gene will not be inhibited by the repressor.

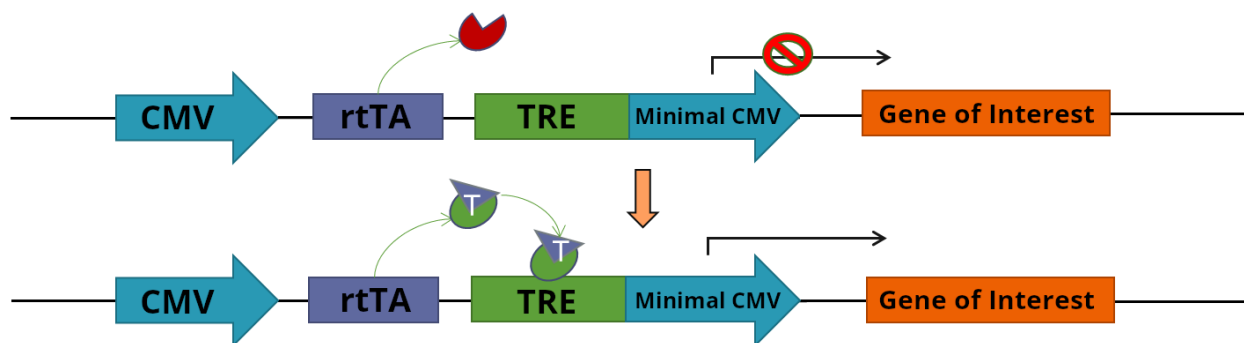
A tetracycline-controlled regulator is either transcriptional activator or repressor. It can bind specifically to TRE, and activate/inhibit the transcription of the downstream gene. A tetracycline-controlled transactivator (tTA) was created by fusing tetR with the C-terminal domain of VP16 (Virion Protein 16), an essential transcriptional activation domain from HSV (herpes simplex virus). In absence of tetracycline, the tetR portion of tTA will bind to the TRE promoter and the activation domain promotes expression. In the presence of tetracycline, tetracycline binds to tetR preventing tTA binding to the TRE promoter and subsequent expression by the gene of interest. This tTA dependent system is also known as tetracycline off (Tet-Off) system (Figure 1).

Figure 1. Schematic diagram of Tet-Off system



Conversely, a tetR mutant was created by random mutagenesis. Several amino acids necessary for tetracyclin-dependent repression have been mutated to reverse the phenotype of tetR, and create dependence on the presence of tetracycline for induction of gene expression, rather than repression. This new tetR mutant was fused with VP16 to create the reverse tetracycline-controlled transactivator (rtTA). The rtTA-dependent system is also known as tetracycline on (Tet-ON) system (Figure 2).

Figure 2. Schematic diagram of Tet-On system



Product Description

OriGene's All-in-one Tet-ON system is a new and improved version of the original Tet-ON systems designed to significantly stimulate expression of the downstream gene of interest (GOI). It has a Tet-ON 3G transactivator and a tightly regulated TRE promoter (P_{TIGHT}) in one vector.

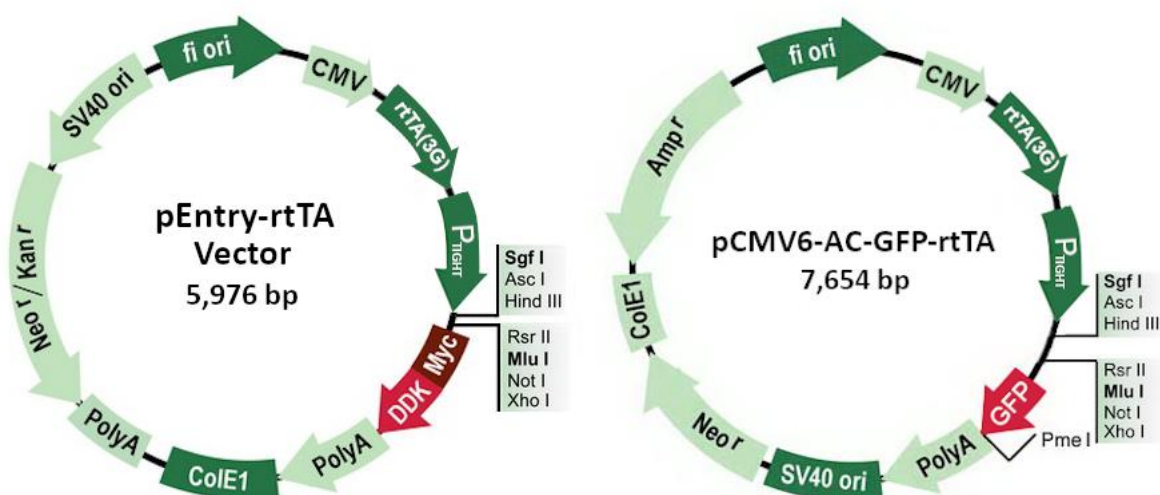
The Tet-ON 3G transactivator consists of a modified bacterial Tet repressor (TetR) fused to three minimal VP16 activation domains to create a transcriptional activator protein. Our Tet-ON 3G transactivator contains mutations that significantly increase its sensitivity to Doxycycline (Dox), a synthetic analog to tetracycline. The increased sensitivity is particularly advantageous for *in vivo* studies in tissues where high Dox concentrations are difficult to attain (e.g., brain).

The tightly regulated P_{TIGHT} promoter consists of the conventional TRE sequence fused upstream of the minimal CMV promoter which provides remarkably low basal activity and high maximal expression after induction. The significantly reduced background expression provides an improved dynamic expression range compared to traditional Tet promoter.

- PS100124 is the C-terminal Myc-DDK tagged Tet-ON inducible vector, consisting of Tet-ON 3G transactivator, tightest TRE promoter (P_{TIGHT}), Multiple Cloning Site (MCS), and C-terminal Myc-DDK tag.
- PS100125 is the C-terminal tGFP tagged Tet-ON inducible vector consisting of Tet-ON 3G transactivator, tightest TRE promoter (P_{TIGHT}), Multiple Cloning Site (MCS), and C-terminal turbo-GFP tag.

View the plasmid maps of PS100124 and PS100125 from figure 3.

Figure 3. The plasmid map of All-in-on Tet-On inducible vectors



Package contents

- 1 vial of 10 μ g lyophilized All-in-one Tet-On inducible vector (SKU PS10024 or SKU PS100025).
- Certificate of Analysis.

Note: The plasmid DNA is shipped at room temperature, but should be kept at -20°C for long-term storage. If properly stored, clones are guaranteed to be stable for 12 months.

Related Optional Reagents

- LB agar plates with kanamycin, 25 $\mu\text{g}/\text{mL}$ (for the amplification of SKU PS100024)
- LB agar plates with ampicillin, 100 $\mu\text{g}/\text{mL}$ (for the amplification of SKU PS100025)
- LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 10 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH)

Related OriGene Products

- Transfection reagent: <https://www.origene.com/products/others/transfection-reagents>
- Antibodies: <https://www.origene.com/products/antibodies>
- qPCR reagents <https://www.origene.com/products/gene-expression/qpcr>

Notice to purchaser

This product is for research use only. Use in and/or for diagnostics and therapeutics is strictly prohibited. By opening and using the product, the purchaser agrees to the following: The plasmids may not be distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from OriGene Technologies, Inc. If you do not agree to the above conditions, please return the UNOPENED product to OriGene Technologies, Inc. within ten (10) days of receipt for a full refund.

Application of Tet-On Vectors

Temporal experiments requiring tight and timely regulation of your target gene expression can be easily accomplished using one of our Tet-ON inducible systems. Because of the improved dynamic expression range of your target, you can reliably test the influence of your target gene in cells or in tissues at specific time points of your interest. Some of the applications where our Tet-ON system could be used for instance to investigate the role of your gene of interest with relation to progression of a disease, cell cycle, or tissue growth. Virtually any experiment requiring regulation of gene expression is easily accomplished with the use of our new and improved Tet-ON system.

Experimental Protocol

Reconstitution & Amplification

The vector is provided as 10 ug lyophilized plasmid DNA. Reconstitute the DNA in 100 μ L dH₂O (final concentration 100 ng/ μ L). Transform 1 ul of the DNA using 20 ul of high efficiency competent E. coli cells (ideally 1×10^8 CFU/ μ g). Following transformation, resuspend cells in 200 uL LB media. Plate the entire transformation reaction on a standard LB-agar plate containing 25ug/ml kanamycin for PS100124 vector and 100ug/ml Ampicillin for PS100125 vector. Incubate at 37°C overnight. Pick at least 4-8 independent colonies to do mini-prep from each transformation. Confirm the insert by restriction digestion and/or vector primer sequencing.

*OriGene offers specific sequencing primers for PS100124 and PS100125 (CAT# PRIMER, including VP1.5 and XL39 primers).

Cloning of OriGene ORF into the Tet-ON inducible Vectors (PS100124 and PS100125)

OriGene offers ORF clones for every human, mouse and rat genes in the pCMV6-Entry (CAT# PS100001) vector, which has compatible cloning sites with all-in-one Tet-ON inducible vectors (CAT# PS100124 or PS100125). To transfer the insert gene from OriGene's ORF clone (donor) into the new inducible vector (recipient), choose the appropriate cloning sites and follow the general cut-and-paste subcloning protocols as below.

1. Digest the donor ORF clone :

Component	Volume
10X restriction buffer	2 μ l
Sgf I (10 U/ μ l)	0.6 μ l
Mlu I (10 U/ μ l)	0.6 μ l
nuclease-free water	13.8 μ l
Donor ORF clone (200 ng)	3 μ l
Total volume	20 μl

Incubate at 37°C for 3 hrs.

2. Digest the inducible Tet-ON destination vector:

Component	Volume
10X restriction buffer	2 μ l
Sgf I (10 U/ μ l)	0.6 μ l
Mlu I (10 U/ μ l)	0.6 μ l
Nuclease-free water	14.8 μ l
Tet-ON destination vector (200ng)	2 μ l
Total volume	20 μl

* For the 4% of the clones that have internal Sgf I or Mlu I sites, please use the appropriate combination of restriction sites as recommended by OriGene

Incubate at 37°C for 3 hrs. Add 0.5 μ l antarctic phosphatase (units used according to the manufacturer's protocol) to the digestion, and continue to incubate at 37°C for an additional 30 minutes.

3. Purify the digestion using a commercial PCR purification column and elute in 20 μ l 10 mM Tris.

4. Set up a ligation reaction:

Component	Volume
10 x T4 DNA ligation buffer	1 μ l
T4 DNA Ligase (4U/ μ l)	0.75 μ l
Nuclease-free water	3.25 μ l
Digested DNA from Step 1 (ORF clone)	2 μ l
Digested DNA from Step 2 (Tet-ON vector)	3 μ l
Total volume	10 μl

5. Incubate the ligation reaction at room temperature for 1 hour.

6. Transform the ligation reaction into high-efficiency, competent *E. coli* cells ($\geq 1 \times 10^8$ CFU/ μ g DNA) following the appropriate transformation protocol. Plate the transformants on LB-agar plates supplemented with either 100 μ g/ml ampicillin for PS100125 or 25 μ g/ml kanamycin for PS100124.
7. Pick at least four colonies for subsequent DNA purification and screening. Amplify and purify the selected clone(s) by growing overnight in liquid LB containing the corresponding antibiotics (ampicillin or Kanamycin), then isolating the DNA using standard plasmid purification procedures.
8. Confirm the insert by restriction digestion and/or vector primer sequencing using the provided V1.5 for 5' end sequencing and XL39 for 3' end sequencing.

Cloning of ORFs into Inducible Tet-ON vector (PS100124 and PS100125) using PCR Amplification

The ORF sequence can be PCR amplified and cloned into the inducible Tet-On vectors (PS100124 and PS100125). In order to append cloning sites to the 5' and 3' ends of the ORF sequence, you need to add the target sequences of the selected restriction enzymes to the forward and reverse PCR primers; examples are shown below.

Forward primer with Sgf I

5' GAGGCGATCGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Ns represent the sequence of the ORF beginning with the start codon, ATG. It is important to add the additional "C" base after the Sgf I site to maintain appropriate reading frames with N-terminal tags in some destination vectors.

Reverse primer with Mlu I

5' GCGACGCGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Ns represent the reverse complement of the ORF sequence starting with the stop codon for N-terminally tagged or untagged destination vectors. This ensures that the expressed fusion protein will end at the native C-terminal end of the ORF. For C-terminally tagged vectors, the reverse complement of the ORF sequence should start with the second-to-last codon, as the stop codon must be removed to generate a fusion protein.

If the recognition sites for Sgf I or Mlu I are present internally in the ORF, another rare cutter such as Asc I, Rsr II or Not I can be used in the cloning strategy. In these cases, the sequences of these alternate restriction sites should be used in place of Sgf I and/or Mlu I (examples below). This same primer design strategy described above should be used for the design of other primers. The Ns in the forward primer represent the sequence of the ORF beginning with the start codon, ATG. The Ns in the reverse primers represent the reverse complement of the ORF sequence starting with the stop codon for N-terminally tagged or untagged destination vectors, or starting with the second- to-last codon for C-terminally tagged vectors.

Forward primer with Asc I:

5' GCCGCGCGCCANNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

An extra nucleotide after Asc I is important to maintain reading frames with N- terminal tags in some destination vectors.

Reverse primer with Rsr II:

5' GCGTCGGTCCGCTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Extra nucleotides after Rsr II are important to maintain appropriate reading frames with C-terminal tags in some destination vectors.

Reverse primer with Not I:
 5' GCGACGCGGCCGCGTACGCGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'
 Mlu I is also added for downstream subcloning.

We recommend using a full-length cDNA clone as the template for ORF cloning. The success rate is low when a cDNA pool is used as the template for a PCR cloning reaction. When the GC content of an ORF (or a region of the ORF longer than 100 bp) is above 75%, a special PCR buffer with DMSO or other additive should be used to increase the success rate. The recommended PCR polymerase and buffer are available from New England Biolabs (Phusion™High-Fidelity PCR Kit, F-553S).

PCR reaction setup:

Component	Volume
5X PCR buffer	4 µl
dNTPs (2.5 mM each)	1.6 µl
Phusion polymerase (2U/µl)	0.2 µl
Nuclease free water	11 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6 µl
cDNA template	2 µl (50-100ng plasmid)
Total volume	20 µl

All of the components should be kept on ice. When setting up multiple reactions, a master mix can be prepared without cDNA template or primers. After aliquoting the master mix, the cDNA template and primers can be added individually to each tube.

PCR cycling conditions:

The optimum T_m for annealing should be 55-60°C. The extension time depends upon the length of the ORF. The following program is generally used for ORFs from 500 bp-4000 bp.

1 cycle of	95°C	1 min
2 cycles of	95°C	10 sec
	62°C	20 sec
	72°C	4 min
2 cycles of	95°C	10 sec
	60°C	20 sec
	72°C	4 min
2 cycles of	95°C	10 sec
	58°C	20 sec
	72°C	4 min
15 cycles of	95°C	10 sec
	56°C	20 sec
	72°C	4 min
Final extension	72°C	10 min
	4°C	hold

Induction and expression testing

Tet-OFF system can be induced by both tetracycline and doxycycline, but the Tet-ON system only responds to the doxycycline (Dox), not tetracycline (Gossen & Bujard, 1995). Also doxycycline is more stable in the culture medium (48 hours versus 24 hours for tetracycline) and appears to be more potent than tetracycline. In vitro 0.01–1.0 µg/ml of Dox has been shown to induce Tet-ON expression within 48 hours. Titration of different Dox concentration, as well as determination of the optimum incubation period to achieve maximal expression, is recommended for each individual target gene.

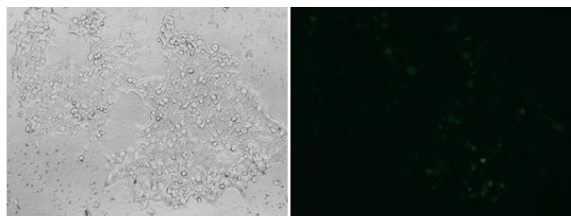
Here, we describe a protocol to test our Tet-On systems (CAT# PS100124 and PS100125). Our test was conducted on HEK 293 cells, however, when using different cell lines, we recommend using this protocol as a reference while taking into consideration cell culture requirements, transfection method, etc., that are appropriate to the host cell line you will be experimenting on.

1. 24 h prior to transfection, 3×10^4 HEK293 cells/well were seeded on to a 96 well plate in growth media consisting of DMEM supplemented with antibiotics and 10% FBS.
2. On the following day, cells were transfected with 0.2 µg of Tet-ON vector (Cat#PS100125) per well using Turbofectin transfection reagent (TF81001, OTI) at 1:3 ratio (DNA:Turbofectin reagent).
3. 24 h post transfection 4 different dosages of Dox (NC0424034, Fisher Scientific) was tested by adding 0.01, 0.1, 1.0, and 10 (µg/ml) in duplicate wells for each dosage respectively. Dox-untreated wells served as control.
4. Post treatment with Dox, Tet-On induced GFP fluorescence in cells was scanned with a fluorescence plate reader on three consecutive days (in case of CAT# PS100124, perform IHC on the transfected cells with Anti-DDK antibody and assay for myc-DDK tag expression). Raw fluorescence readouts expressed in arbitrary units (a.u.) for each dosage of Dox treatment was compared to the control group (Dox-untreated) to determine the induction efficiency of our Tet-ON system (CAT# PS100125).

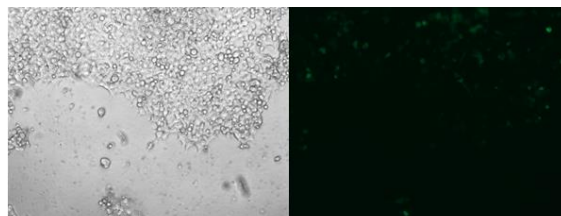
Results

Significant induction of GFP expression was observed in HEK 293 cells transfected with Tet-On vector (Cat#PS100125) at Dox dosage of 1µg/ml or higher, 72 hours post treatment, as shown in the microscopic images in Figure 4. Concomitantly, we performed a quantitative measurement of the induction of the GFP expression by Dox on the same cells using a fluorescence plate reader at 0, 24, 48, and 72 hours post Dox treatment (Figure 5). Raw fluorescence intensity readout (expressed in arbitrary units) from the plate reader plotted against varying concentrations of Dox within their respective days clearly demonstrates the most effective dosage to be at 1µg/ml or higher which reflects the observation seen in the microscopic images in Figure 4.

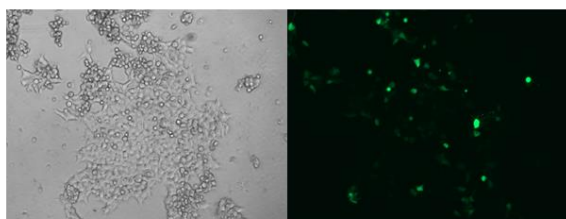
Figure 4. Representative microscopic images of GFP expression as a measure of induction of our Tet-ON system (PS100125) by Doxycyclin (Dox)



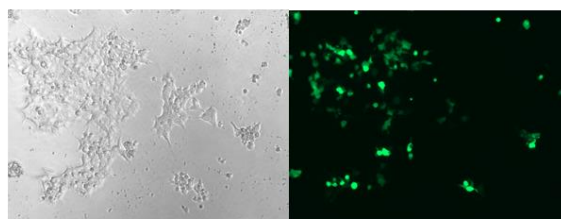
Tet-On Vector with 0 ng/mL Dox



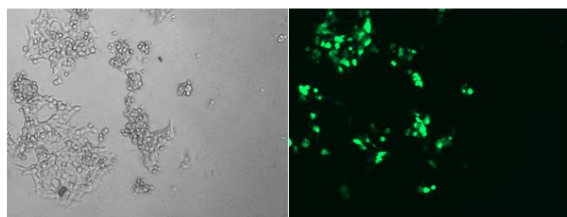
Tet-On Vector with 10 ng/mL Dox



Tet-On Vector with 100 ng/mL Dox



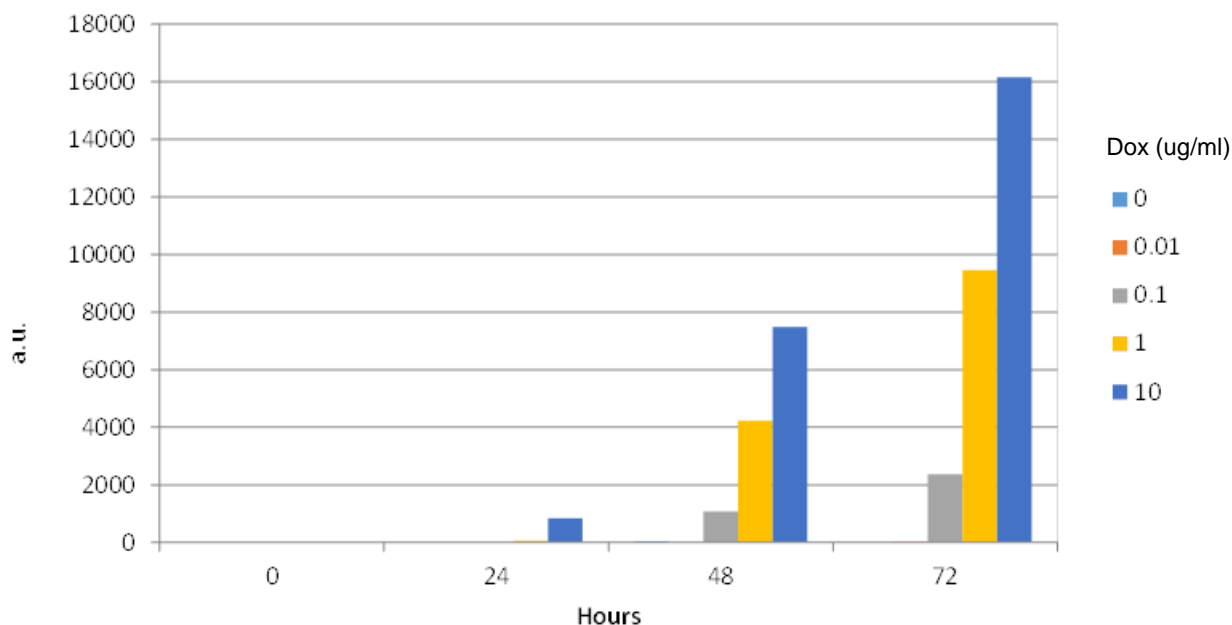
Tet-On Vector with 1 ug/mL Dox



Tet-On Vector with 10 ug/mL Dox

Representative microscopic images of GFP expression as a measure of induction of our Tet-ON system (PS100125) by Dox at varying concentration of 0, 0.01, 0.1, 1, 10 (ug/ml). Significant GFP expression is easily identifiable in cells induced by Dox at 100 ug/ml or higher. Along with the fluorescent images are bright field views of the cells in culture.

Figure 5. Dose response induction of Tet-ON system (CAT# PS100125) with Dox as measured by the fluorescence intensity of GFP



A 96 well plate fluorescence reader was used to assess the induction of the Tet-ON system by Dox by measuring the GFP expression at 0, 24, 48, and 72 hours post induction. Cells were treated with 0, 0.01, 0.1, 1, 10 (ug/ml) of Dox. GFP intensity readout from the plate reader was plotted on the y-axis (arbitrary units, a.u.) against cells treated with varying concentration of Dox on the x-axis at different time points. Maximal induction of our Tet-ON system (PS100125) was observed at 72 hours post Dox treatment for all doses of Dox whereas virtually none was quantified in cells that was not induced by the Dox.

Reference

Gossen, M. & Bujard, H. (1995) Efficacy of tetracycline-controlled gene expression is influenced by cell type. *BioTechniques* 89:213–215