Clontech Laboratories, Inc.

AAVpro® Tet-One™ Inducible Expression System (AAV2) User Manual

Cat. No. 634310
(021215)

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I. Introduction

A. Summary

The **Tet-One Systems** are inducible gene expression systems for mammalian cells that contain all the necessary components in a single plasmid, lentiviral, retroviral, or adeno-associated viral (AAV) vector. After introducing the plasmid (Tet-One Systems) to target cells, the cells will express the Tet-On® 3G transactivator protein and contain a gene of interest (GOI) under the tight control of a TRE3G promoter (P_{TRE3GS}). This user manual describes the AAV-based **AAVpro Tet-One Inducible Expression System** (Cat. No. 634310). Using this system, your target cells or tissues will express high levels of your GOI, but only when cultured in the presence of doxycycline (Dox) (Figure 1).

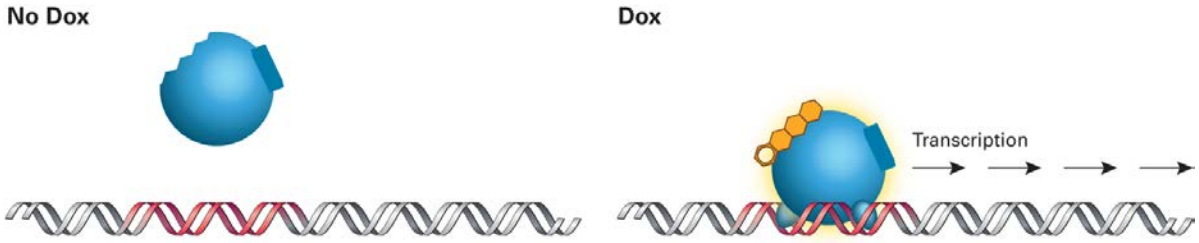

Figure 1. The Tet-One System allows inducible gene expression in the presence of Dox. The region in red represents the TRE3G promoter.

B. Elements of the AAVpro Tet-One System

Tet-On 3G Transactivator Protein

Based on the transcriptional regulators described by Gossen & Bujard (1992), Gossen *et al.* (1995), and Urlinger *et al.* (2000), Tet-On 3G is a modified form of the Tet-On Advanced transactivator protein which has been evolved to display far higher sensitivity to Dox (Zhou *et al.*, 2006).

P_{TRE3GS} Inducible Promoter

The inducible promoter P_{TRE3G} provides for very low basal expression and high maximal expression after induction (Loew *et. al.*, 2010). It consists of 7 repeats of a 19-bp tet operator sequence located upstream of a minimal CMV promoter. P_{TRE3GS} is a version of P_{TRE3G} that was modified for higher performance in a single vector context. In the presence of Dox, Tet-On 3G binds specifically to P_{TRE3GS} and activates transcription of the downstream GOI. P_{TRE3GS} lacks binding sites for endogenous mammalian transcription factors, so it is virtually silent in the absence of induction.

Tet-One Systems "All-in-One" Design

Before the Tet-One Systems were developed, Clontech's Tet-On and Tet-Off® products all required two separate vectors to introduce the transactivator protein and the inducible promoter controlling your GOI, respectively, into your target cells. The Tet-One Systems provide both of these components on a single vector. The Tet-On 3G transactivator is expressed in the reverse direction from the human phosphoglycerate kinase 1 promoter, and the cloned GOI is expressed from the P_{TRE3GS} promoter in the forward orientation. Compared to the two-vector Tet-On 3G Systems, all previously published all-in-one vectors have shown a low signal-to-noise ratio, typically providing only 50–100-fold induced expression, even in selected clones. Clontech's Tet-One Systems are based on an all-in-one design that has shown up to 25,000-fold induction (Heinz *et. al*, 2011).

AAVpro Packaging System

The AAVpro Helper Free System (AAV2) (sold separately, Cat. No. 6230) is a unique system for the preparation of high-titer AAV particles without the use of a helper virus. Use of human microRNA, miR-342, can increase titer by approximately 2-fold as compared to ordinary pRC2 vectors that express only Rep and Cap (refer to the AAVpro Helper-Free System User Manual at **www.clontech.com/manuals**).

AAV Particle Extraction Solution

Extraction of AAV particles from AAV-producing cells is conventionally performed by freeze-thaw or sonication methods; however, these methods are time consuming and require special equipment. This kit includes AAVpro Extraction Solution that allows simple and efficient AAV particle isolation while minimizing protein and nucleic acid contamination. The AAVpro Extraction Solution (Cat. No. 6235) can also be purchased separately.

C. Doxycycline

Doxycycline is a synthetic tetracycline derivative that is the effector molecule for all Tet-On and Tet-Off Systems. When bound by Dox, the Tet-On 3G protein undergoes a conformational change that allows it to bind to *tet* operator sequences located in the P_{TRE3GS} promoter (Figure 1). The Dox concentrations required for induction are far below cytotoxic levels for either cell culture or transgenic studies, and Tet-On 3G responds to even lower concentrations than its predecessors (Zhou *et al.*, 2006). Note that Tet-On and Tet-One Systems respond well only to doxycycline, and not to tetracycline (Gossen & Bujard, 1995). The half-life of Dox in cell culture medium is 24 hr. To maintain continuous inducible GOI expression in cell culture, the medium should be replenished with Dox every 48 hr.

II. List of Components

Store all vectors at –20°C. Store AAV Extraction Solution A and AAV Extraction Solution B at room temperature after thawing. Use within 2 years of receipt.

AAVpro Tet-One Inducible Expression System (Cat. No. 634310)

- AAVpro Tet-One Vector Set (Cat. No. 634311; not sold separately)
  - 20 µl pAAV-TetOne Vector (500 ng/µl)
  - 20 µl pAAV-TetOne-Luc Control Vector (500 ng/µl)
- pRC2-mi342 Vector (Cat. No. 6281; not sold separately)
  - 20 µl pRC2-mi342 Vector (1 µg/µl)
- pHelper Vector (Cat. No. 6282; not sold separately)
  - 20 µl pHelper Vector (1 µg/µl)
- AAVpro Extraction Solution (Cat. No. 6283; not sold separately)
  - 3 x 1.5 ml AAV Extraction Solution A
  - 3 x 150 µl AAV Extraction Solution B

III. Additional Materials Required

The following reagents are required but not supplied.

A. Tetracycline-Free Fetal Bovine Serum

Contaminating tetracyclines, often found in serum, will significantly elevate basal expression when using Tet-On 3G. The following functionally tested tetracycline-free sera are available from Clontech:

Cat. No.	Serum Name
631106	Tet System Approved FBS (500 ml)
631107	Tet System Approved FBS (50 ml)
631101	Tet System Approved FBS, US-Sourced (500 ml)
631105	Tet System Approved FBS, US-Sourced (50 ml)

B. Mammalian Cell Culture Supplies

- **Medium for HEK 293 or HEK 293T Cells:**
  Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4.5 g/L), 4 mM L-glutamine, and sodium bicarbonate (Sigma-Aldrich, D5796); 10% Fetal Bovine Serum (FBS); 1% Antibiotic Antimycotic Solution (AAS; Sigma-Aldrich, A5955)
- Culture medium, supplies, and additives specific for your target cells
- 0.5 M EDTA (pH8.0)
- 6-well, 12-well, 24-well or 96-well cell culture plates, and 10-cm cell culture dishes

C. In-Fusion® HD Cloning System

The In-Fusion cloning system is a ligation-free technology that greatly simplifies cloning.

Cat. No.	In-Fusion Cloning Kit
638909	In-Fusion HD Cloning Plus (10 rxns)
638910	In-Fusion HD Cloning Plus (50 rxns)
638911	In-Fusion HD Cloning Plus (100 rxns)

D. Stellar™ Competent Cells

Stellar Competent Cells are recommended by Clontech for cloning of viral vectors. Propagation of vectors containing repeat sequences such as viral ITRs or LTRs using other strains of *E.coli* may result in plasmid rearrangements. Stellar Competent Cells are sold separately and are provided with all In-Fusion Cloning Systems.

Cat. No.	Competent Cells
636763	Stellar Competent Cells (10 x 100 µl)
636766	Stellar Competent Cells (50 x 100 µl)

E. Plasmid Purification (Transfection-Grade)

Cat. No.	Product	Size
740412.10	NucleoBond Xtra Midi Plus	10 preps
740414.10	NucleoBond Xtra Maxi Plus	10 preps

F. Xfect™ Transfection Reagent

We recommend using Xfect reagent for transfection. Xfect reagent provides high transfection efficiency for most commonly used cell types, including HEK 293 and HEK 293T cells.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

G. AAV Titer Determination

For accurate and consistent transductions, we highly recommend titrating your AAV stocks using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233).

H. AAV Purification

The AAVpro Purification Kit (AAV2) (Cat. No. 6232) can be used to further purify AAV particles for transduction into individual animals and cultured cells.

I. Doxycycline

- 5 g Doxycycline (Cat. No. 631311)

Dilute to 1 mg/ml in double distilled H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.

J. TetR Monoclonal Antibody

If you wish to confirm that Tet-On 3G is expressed in your cells, we recommend that you use the following antibody and detect the protein via Western Blot.

Cat. No.	Antibody
631131	TetR Monoclonal Antibody (Clone 9G9) (40 µg)
631132	TetR Monoclonal Antibody (Clone 9G9) (200 µg)

K. Luciferase Assay and Luminometer

These items are required when using the pAAV-TetOne-Luc Vector as a control to test for induction (Section VI.B). Use any standard firefly luciferase assay system and luminometer.

IV. Protocol Overview

Please read each protocol completely before starting. Successful results depend on understanding and performing the following steps correctly.

A. General Cell Culture

This user manual provides only general guidelines for mammalian cell culture techniques. For users requiring more information on mammalian cell culture and transfection, we recommend the following general reference:

Freshney, R.I. (2005). *Culture of Animal Cells: A Manual of Basic Technique, 5th Edition* (Wiley-Liss, Hoboken, NJ).

B. Safety Guidelines for Working with Recombinant AAV

The protocols in this manual require the handling of AAV vectors. It is imperative to fully understand the potential hazards of and necessary precautions for laboratory use of these vectors. Viruses produced with AAV-based vectors could, depending on your gene insert, be potentially hazardous. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*. For these reasons, due caution must be exercised in the production and handling of any recombinant viruses. Follow all applicable guidelines for research involving recombinant DNA. Take appropriate safety measures when producing or handling recombinant AAV, including working in a biological safety cabinet and wearing protective laboratory coats, face protection, and gloves.

C. Protocol Summary

The following are the steps required to create a Dox-inducible expression system using AAVpro Tet-One (see Figure 2).

1. Clone your gene of interest into the pAAV-TetOne Vector using the In-Fusion HD Cloning System (Section V).
2. Pilot test Tet-based induction of your construct using transient transfection (Section VI).
3. Produce AAV particles using the AAVpro Helper-Free System (Section VII).
4. Transduce your target cells with the Tet-One AAV virus (Section IX).

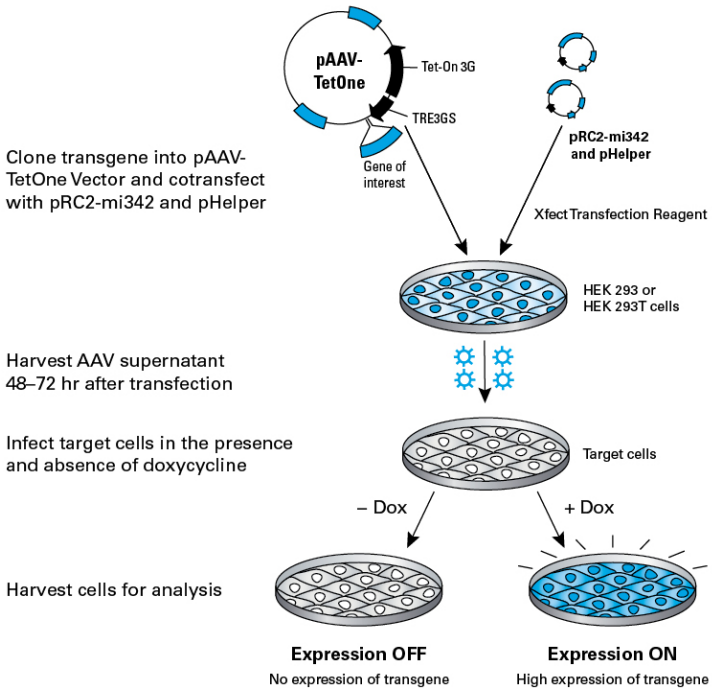

Figure 2. Establishing an inducible expression system in target cells with AAVpro Tet-One. The pAAV-TetOne Vector containing your gene of interest, pRC2-mi342, and pHelper are cotransfected into HEK 293 or HEK 293T cell lines to generate a high-titer AAV particles (Section VII). The AAV particles are used to transduce your target cells and Dox is added to express your gene of interest when desired (Section IX).

V. Cloning Your Gene of Interest (GOI) into a pAAV-TetOne Vector

1. Insert your GOI into the multiple cloning site (MCS) of the pAAV-TetOne Vector.

   We recommend using In-Fusion cloning for generating pAAV-TetOne vectors containing your GOI. Follow the protocol outlined in the In-Fusion HD Cloning Kit User Manual available at **www.clontech.com/manuals**.

   NOTE: The GOI DNA fragment should contain an ATG start codon and a stop codon. pAAV-TetOne Vector allows for a transgene up to 2.5 kb to be cloned. However, virus titer and/or expression level may decrease when the transgene is larger than 2 kb.

   The recommended linearization sites and forward/reverse primer design recommendations for In-Fusion cloning are shown below. The example primers below have a 20-bp region that anneals to the target GOI sequence.

Vector	Linearize with	Forward Primer*	Reverse Primer**
pAAV-TetOne	EcoRI & NotI	CCCTCGTAAAGAATTC 111 222 333 444 555 666 77	TCCACGCGTGCGGCCGC SSS NNN NNN NNN NNN NNN NN

*111 = Start codon of your gene; 222 = 2nd codon of your gene; etc.

**SSS = reverse complement of the stop codon of your gene; NNN = reverse complement of the end of your gene.

NOTE: Stellar Competent Cells (Section III.D) are recommended by Clontech for cloning of viral vectors. Propagation of vectors containing repeat sequences such as viral ITRs or LTRs using other strains of *E. coli* may result in plasmid rearrangements. Stellar Competent Cells are provided with all In-Fusion HD Cloning Systems.

2. After confirming the presence of the correct insert (pAAV-TetOne-GOI), prepare transfection-grade plasmid DNA using a plasmid purification kit, such as NucleoBond Xtra Midi/Maxi (Cat. No. 740410.10/740414.10, etc.). Adjust the plasmid DNA concentration to 1 µg/µl.

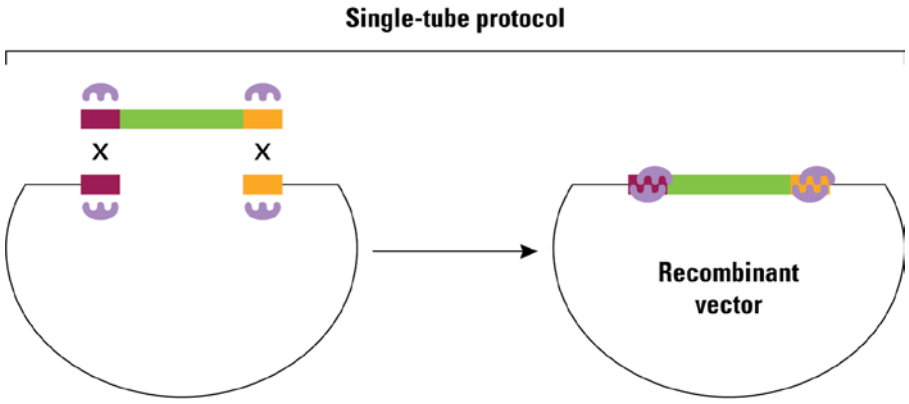

Figure 3. The In-Fusion single-tube cloning protocol.

VI. Pilot Testing Tet-Based Induction of Your Construct

A. General Considerations

Prior to AAV production, your pAAV-TetOne construct should be tested for functionality by plasmid transfection. Transiently transfect your vector into an easy-to-transfect cell line such as HeLa or HEK 293, or your target cell line, and test for transgene induction with Dox. You will need an appropriate gene-specific assay to test for induction, such as:

- Western blot
- Northern blot
- qRT-PCR
- Gene-specific functional assay
- A plasmid that expresses your GOI from a constitutive promoter (e.g., CMV promoter) can be used as a positive control.

B. Protocol

1. Transfect the TetOne vector into your target cells (in a 6-well plate) using Xfect Transfection Reagent. Follow the **Xfect Transfection Reagent Protocol-At-A-Glance** available at **www.clontech.com/manuals**.
   - Use 5 µg of pAAV-TetOne, pAAV-TetOne-GOI, and a positive control plasmid for each well; prepare duplicate wells
   - Add Dox (100 ng/ml) to one set of wells (induced samples)

Well 1: 5 µg pAAV-TetOne (no Dox)

Well 2: 5 µg pAAV-TetOne (100 ng/ml Dox)

Well 3: 5 µg pAAV-TetOne-GOI (no Dox)

Well 4: 5 µg pAAV-TetOne-GOI (100 ng/ml Dox)

Well 5: 5 µg positive control plasmid (no Dox)

Well 6: 5 µg positive control plasmid (100 ng/ml Dox)

Figure 4. Transfection of the pAAV-TetOne vectors into target cells in a 6-well plate.

2. After 24 hr, harvest the cell pellets from each well and compare induced expression levels to uninduced expression levels using a method appropriate for your GOI.

VII. Producing AAV

A. General Considerations

- Several HEK 293 and HEK 293T cell lines are commercially available, but some lines are unable to generate high-titer AAV. Once a HEK 293 cell line that results in high-titer AAV is identified, use that cell type consistently. HEK293T/17 cells (ATCC, CRL-11268) are recommended for preparation of high-titer AAV.
- We recommend using Tet System Approved FBS. Subsequent Tet-One System performance will be negatively affected by serum containing tetracycline contaminants (see Section III.A).

B. Protocol

NOTE: This protocol can be completed in 3–4 days.

1. Transfect HEK 293 or HEK 293T Cells with the AAVpro Vectors

a) Seed HEK 293 or HEK 293T cells in a 100-mm plate in 10 ml of DMEM/10%FBS (use Tet System Approved FBS)/1% Antibiotic Antimycotic Solution, and incubate at 37°C, 5% CO_2. Plate at a density such that the cells will be 50–80% confluent at the time of transfection.

b) Thaw and thoroughly vortex Xfect Polymer.

c) For each transfection sample, prepare the transfection mix in a microcentrifuge tube by adding the following reagents:

561 µl	Xfect Reaction Buffer
13 µl	pAAV-TetOne Vector containing GOI (1 µg/µl)
13 µl	pRC2-mi342 Vector (1 µg/µl)
13 µl	pHelper Vector (1 µg/µl)
600 µl	Total Volume

d) Vortex 5 seconds to mix thoroughly.

e) Add 11.7 µl of Xfect Polymer to the plasmid mixture, and vortex vigorously for 10 seconds to mix.

f) Spin down for 1 sec to collect the contents at the bottom of the tube and incubate for 10 min at room temperature to allow nanoparticle complexes to form.

g) Add the entire DNA-Xfect solution dropwise to the cell culture medium. Rock the plate gently back and forth to mix.

NOTE: It is <u>not</u> necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the DNA-Xfect solution.

h) Incubate the plate at 37°C, 5% CO_2.

i) After at least 6 hr, replace the transfection medium with 10 ml fresh DMEM/2% FBS (use Tet System Approved FBS)/1% Antibiotic Antimycotic Solution and incubate at 37°C 5% CO_2 for 2–3 days.

2. Isolate AAV Particles

a) Add 125 µl of 0.5 M EDTA (pH 8.0) to the 10-ml culture (1/80 volume). Rock the plate back and forth to thoroughly mix and allow to stand at room temperature for 10 min.

b) Collect the medium containing the detached cells in a sterile 15-ml centrifuge tube.

c) Centrifuge at 1,750 x *g* for 10 min at 4°C and completely remove the supernatant.

NOTE: Confirm that the supernatant has been completely removed before proceeding; particle isolation may be affected by residual supernatant.

d) Loosen the cell pellet by tapping or vortexing the tube.

e) Add 0.5 ml of AAV Extraction Solution A and vortex for 15 sec to suspend the cells.

f) Allow to stand at room temperature for 5 min.

g) Vortex for 15 sec and centrifuge at 2,000–14,000 x *g* for 10 min at 4°C to remove cell debris.

h) Collect the supernatant in a new sterile microcentrifuge tube, add 50 µl of AAV Extraction Solution B, and mix.

NOTE: The mixture can be used for viral titer determination using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) or for transduction of target cells. Otherwise, the mixture can be stored at –80°C until use. When necessary, thaw quickly in a 37°C water bath before use.

VIII. Determining AAV Titer

A. General Considerations

Determining the viral titer is necessary to obtain the following information:

- Confirmation that virus was produced.
- The proper transduction conditions for your particular cell type based on the multiplicity of infection (MOI) for the desired transduction efficiency (MOI = No. of infectious virus particles per target cell). An excessively high MOI can be toxic to cells while an extremely low MOI may not enable you to accurately evaluate the phenotype of infected cells.
- The maximum number of target cells that can be transduced by a given virus volume.

B. Protocols

Virus titer can be measured by real-time PCR (vector genome assay) or by infection assay (biological titer measurement). Real-time PCR analysis of vector genomes provides rapid quantification, whereas determining titer by infection of cells is generally more accurate. There are other titration methods for AAV2 vectors that involve assay of viral capsid proteins, but these methods may detect nonfunctional (empty) particles.

1. Vector Genome Assay

The AAVpro Titration Kit (for Real Time PCR) (Cat. No. 6233) can be used to measure virus titer by real-time PCR analysis using the viral ITR domain as a target.

2. Biological Titer Measurement Method

Determine titer by measuring the expression of the gene of interest. A protocol for quantification of an AAV2 vector expressing the fluorescent protein ZsGreen1 (pAAV-ZsGreen1 Vector, Cat. No 6231) is provided below.

a) Prepare HT1080 cells at a concentration of 4 x 10^4 cells/ml in DMEM/10% FBS/1% Antibiotic Antimycotic Solution.
b) Inoculate several wells of a 24-well plate with 0.5 ml of the cell suspension (2 x 10^4 cells) and culture overnight.
c) Prepare serial dilutions of the prepared AAV2 particle solution DMEM/10% FBS/1% Antibiotic Antimycotic Solution. The dilution ratio depends on the virus titer, but serial dilutions in the 1,000–100,000-fold range are recommended.
d) Three days after infection, detach the cells using Trypsin/EDTA, and analyze ZsGreen1 expression by flow cytometry.

IX. Transducing Target Cells and Inducing GOI Expression with the Tet-One AAV

A. General Considerations

Successful transduction and induction of your GOI depends on the type of target cells or tissues used. Therefore, optimization of experimental conditions will be required.

B. Example Protocol: Transduction and Induction in Dividing Cells

NOTE: This protocol can be completed in 2–4 days.

1. Prepare enough plates/wells for target cells in complete growth medium 12–18 hr before transduction. The seeding density will depend on the growth characteristics of your cell line.
2. Thaw your AAV stock, or use virus freshly prepared from packaging cells (Section VII).
3. Transduce your target cells using several MOIs in duplicate. We recommend testing 1 x 10^4–1 x 10^6 MOI (genomic titer) at first (Figure 5). Rock the plate gently back and forth to spread the virus evenly.

   NOTE: Make sure that the total volume of viral extract used is no more than 1/10 of the culture medium volume. Contaminants in the AAV extract may be toxic to the cells. Alternatively, the particles can be purified using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).

4. Add an appropriate amount of Dox to half of the transduced cells to induce expression of your GOI at the desired time.

   NOTE: For most experiments, 100 ng/ml of Dox will induce high levels of expression (Figure 6). You can also determine the appropriate amount required for your experiment. In general, very high MOIs will yield higher maximal expression in the presence of Dox, but also will lead to higher background expression in the absence of Dox.

5. Continue to incubate and analyze the cells at appropriate time points (i.e., 24–72 hr, to allow the expressed protein to accumulate) (Figure 7).

   NOTE: It is not necessary to remove the virus from the medium, but it may be necessary to add additional Dox after 48 hr.

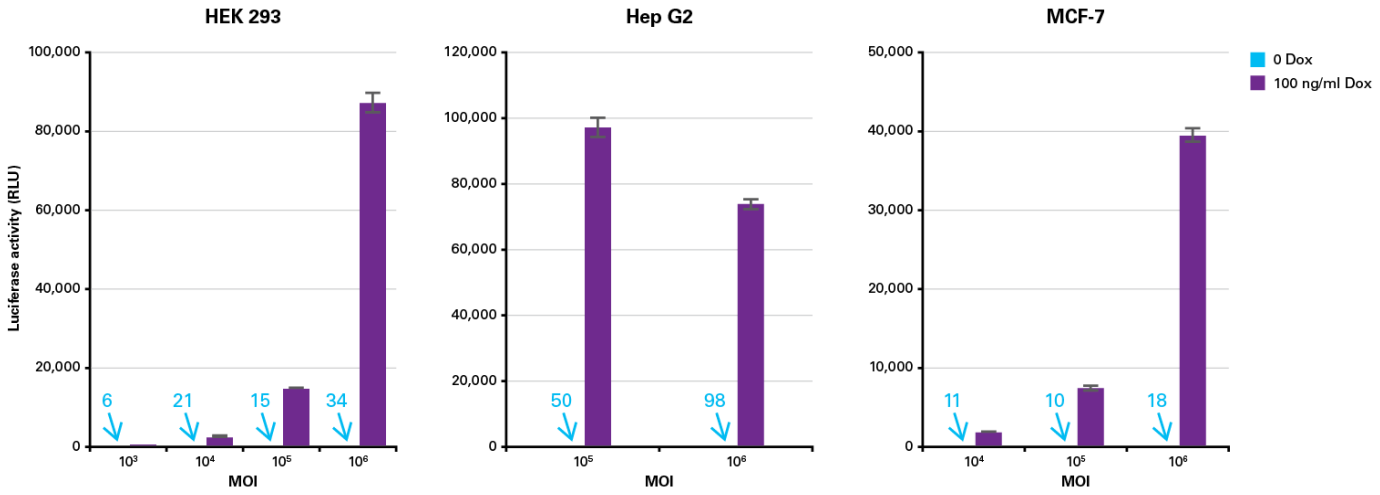

Figure 5. Transduction and induction in dividing cell lines with different MOIs of TetOne-Luc Control AAV. 1 x 10^5 cells were seeded in 12-well plates 12 hr before transduction. Cells were transduced with different MOIs (genomic titer), and 100 ng/ml Dox was added. After 48 hr, cells were harvested and analyzed with a standard firefly luciferase assay using a luminometer.

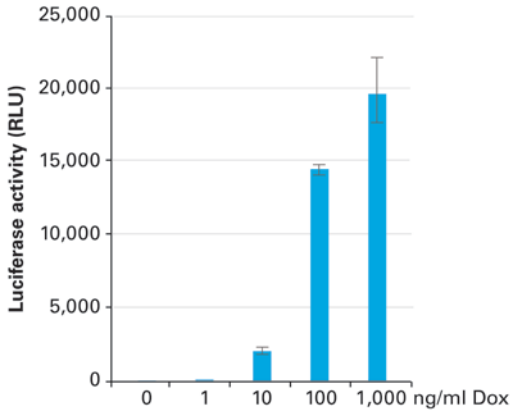

Figure 6. Induction of luciferase after transduction with TetOne-Luc Control AAV in the presence of different concentrations of Dox. 1 x 10^5 HEK 293 cells were seeded in 12-well plates 12 hr before transduction. Cells were transduced with 1 x 10^5 MOI (genomic titer), and Dox was added at different concentrations. After 48 hr, cells were harvested and analyzed with a standard firefly luciferase assay using a luminometer.

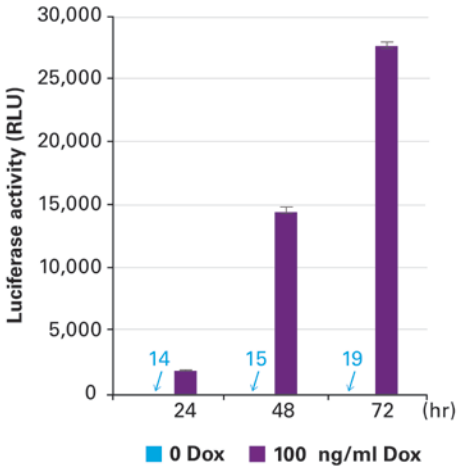

Figure 7. Different induction times of luciferase after transduction with TetOne-Luc Control AAV. 1 x 10^5 HEK 293 cells were seeded in 12-well plates 12 hr before transduction. Cells were transduced with 1 x 10^5 MOI (genomic titer), and 100 ng/ml Dox was added. After 24, 48, and 72 hr, cells were harvested and analyzed with a standard firefly luciferase assay using a luminometer.

X. References

Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH) and in Dr. Wolfgang Hillen's laboratory at the University of Erlangen, Germany. Additional background information on Tet-regulated gene expression systems and an extensive bibliography are available at the website maintained by TET Systems: http://www.tetsystems.com (Please note that Clontech is not responsible for the information contained on this website.)

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Zhou, X., Vink, M., Klave, B., Berkhout, B. & Das, A. T. (2006) Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther.* **13**(19):1382–1390.

Appendix A. Troubleshooting Guide

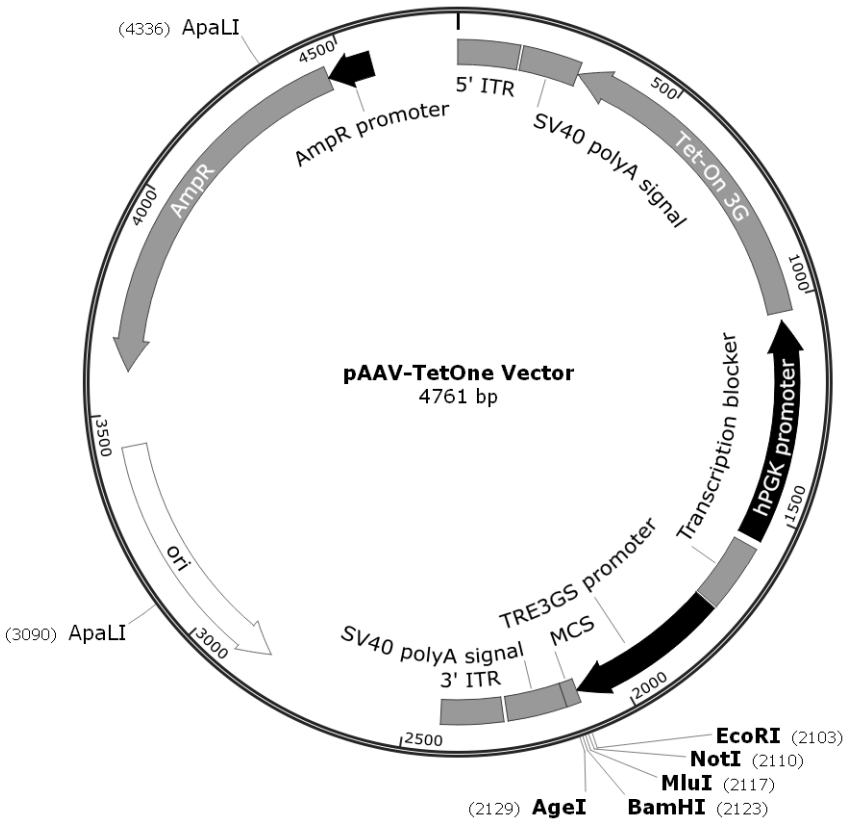
Table 1. Troubleshooting Guide for the AAVpro Tet-One Inducible Expression System (AAV2)

Problem	Possible Explanation	Solution
A. Vector Cloning		
Plasmid is difficult to grow or clone	Some viral vectors may undergo rearrangement between the 5' and 3' ITRs/LTRs when propagated in less-than-optimal *E. coli* host strains	Use Stellar Competent Cells (Cat. No. 636763) to produce high DNA yields and to minimize the potential for DNA rearrangements.
B. Virus Production		
Poor transfection efficiency	Cells plated too densely or not densely enough	Adjust seeding density such that cells are 50–80% confluent at the time of transfection.
	Cell characteristics have changed	Use cells at a low passage.
	Purity of plasmid is not a transfection-grade	Prepare plasmid DNA using a plasmid purification kit, such as NucleoBond Xtra Midi/Maxi (Cat. No. 740410.10/740414.10, etc.).
Low titer	Poor transfection efficiency	See above.
	Virus was harvested too early or too late	Harvest virus 2–3 days after transfection.
	Transfection is toxic to cells	Use the optimized conditions provided in Section VII.
	Cloned transgene is too large	Reduce the size of the transgene to be cloned as much as possible. pAAV-TetOne allows for a transgene up to 2.5 kb, but the virus titer may decrease when the transgene is larger than 2 kb.

Problem	Possible Explanation	Solution
C. Transduction of Target Cells		
Poor transduction efficiency	Target cells are not susceptible to infection by AAV2	Test infectivity of target cells using the virus prepared with pAAV-ZsGreen1 Vector (Cat. No. 6231).
	Too little virus	Determine an exact virus titer using the AAVpro Titration Kit (for Real Time PCR) Ver. 2 (Cat. No. 6233) and use a higher MOI.
	Virus extract contains transduction inhibitors	Purify your virus prior to transduction using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).
	Cells plated too densely or not densely enough	Optimize density of cells when transducing.
Poor target cell viability	Culture conditions are not appropriate	Optimize the culture conditions for the target cells.
	Virus extract contains toxic substances	Purify your virus using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).
	MOI is too high	Infect at a lower MOI.
	Excess viral extract	Use no more than 1/10 of the culture medium. Alternatively, the particles can be purified and concentrated using the AAVpro Purification Kit (AAV2) (Cat. No. 6232).
D. Inducing Expression		
Low fold induction (ratio of maximal to basal expression of the GOI)	Poor transduction efficiency	See section C.
	Poor target cell viability	See section C.
	Cells were analyzed too soon or too late	Analyze cells 24–72 hr after addition of doxycycline.
	The FBS used in the cell culture medium contains tetracycline derivatives	Use Clontech's Tet System Approved FBS (Section III.A), which was functionally tested with Clontech's double-stable CHO-AA8-Luc Tet-Off Control Cell Line.
Decrease in fold induction over time	AAV genome copy number decreases	Optimize the time frame for analyzing GOI expression in target cells.
	Doxycycline was degraded	Add additional doxycycline after 48 hr.

Appendix B: AAVpro Tet-One System Vector Information

For complete descriptions of the vectors provided with each system, refer to the Certificate of Analysis, which is available at www.clontech.com.



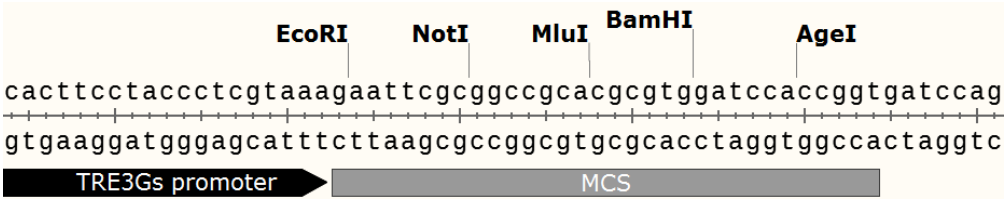


Figure 8. pAAV-TetOne Vector map and multiple cloning site (MCS)

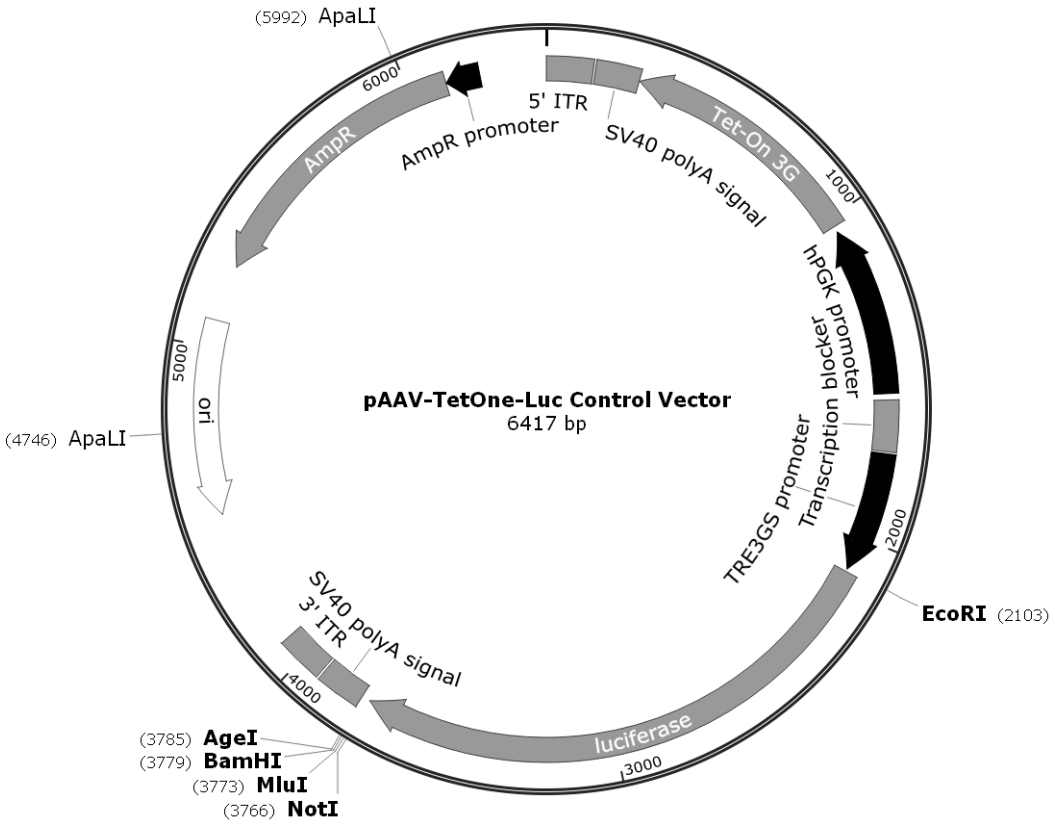


Figure 9. pAAV-TetOne-Luc Control Vector map

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This document has been reviewed and approved by the Clontech Quality Assurance Department.