User Manual

Knockout™ Inducible RNAi Systems User Manual

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I. Introduction

A. Summary

The human genome project has generated the sequences of thousands of genes (Aaronson *et al.*, 1996; Hillier *et al.*, 1996), allowing researchers to focus on the question of gene function in biology. A key approach to determining gene function has been the targeted "knockout" of specific genes; gene inactivation is accomplished by disruption of the target gene's coding sequence and then introduction of the altered gene into embryonic stem cells. Animal models carrying heterozygous and homozygous gene knockouts allow the determination of whether a particular gene is essential and what functions are perturbed by its loss. However, the amount of time and labor required to generate animal knockout models is quite extensive. In addition, achieving such models in somatic cell lines has proven difficult (Sedivy & Dutriaux, 1999).

Another method for eliminating gene expression takes advantage of the phenomenon of post-transcriptional gene silencing (PTGS). Specifically, the cellular process of RNA interference (RNAi) has been used to effectively silence gene expression (Figure 1). RNAi is activated by introducing a double-stranded (ds) RNA whose sequence is homologous to the target gene transcript.The exogenous RNA is digested into 21–23 nucleotide (nt) small interfering RNAs (siRNAs), which bind a nuclease complex to form an RNA-induced silencing complex (RISC). The RISC then targets endogenous gene transcripts by base-pairing and cleaving the mRNA (Hammond *et al.*, 2001; Sharp, 2001; Huntvagner & Zamore, 2002; and Nykanen *et al.*, 2001). In contrast to traditional knockout methods, specific gene silencing is achieved quickly and easily in both animal and cell line models.

The **Knockout™ Inducible RNAi Systems** allow you to quickly express functional hairpin siRNA molecules with tight on/off regulation in mammalian cells for the purpose of silencing target genes.These systems contain RNAi-Ready pSIREN-RetroQ-Tet and tTS expression vectors that use the cell's own RNA Polymerase III (Pol III) to transcribe a specifically designed small hairpin RNA (shRNA) under the control of a hybrid promoter that contains aTet-responsive element and the human U6 promoter.The human U6 promoter provides a high level of expression in many cell types (Kunkel & Pederson, 1989), resulting in target gene suppression.

For maps and detailed information on the pSIREN-RetroQ-TetH Vector and pSIREN-RetroQ-TetP Retroviral Vectors, see the Appendix or the Vector Information Packet(s) provided with your product.

I. Introduction *continued*

B. Mechanism of RNAi

The current model of the RNAi mechanism includes both initiator and effector steps (for reviews see Hutvagner & Zamore, 2002; Hammond *et al.*, 2001; and Sharp, 2001). The initiator step involves the digestion of the input dsRNA into siRNAs 21–23 nt in length. These siRNAs are produced by the action of an enzyme known as Dicer, which belongs to the RNAse III family of dsRNA-specific ribonucleases and is evolutionarily conserved in worms, plants, fungi, and mammals (Bernstein *et al.*, 2001). The cleavage of input dsRNA by Dicer is accomplished in a processive, ATP-dependent manner, eventually generating 19–21 bp siRNA duplexes with a 3' overhang of 2 nt (Figure 1).

The effector step occurs when these siRNA duplexes bind to a nuclease complex and form the RISC (Figure 1). RISC is activated by the ATP-dependent unwinding of the siRNA duplex. Active RISC then targets the native, homologous transcript by base pairing and subsequently cleaving the mRNA at approximately 12 nt from the 3' end of the siRNA (Hammond *et al.*, 2001; Sharp, 2001; Huntvagner & Zamore, 2002; and Nykanen *et al.*, 2001). An amplification step has also been proposed to explain the potency of the RNAi process; the exogenous RNA is copied many times either before or after the generation of the siRNAs (Hammond *et al.*, 2001; Sharp, 2001; and Huntvagner & Zamore, 2002).

RNAi, then, can serve as a powerful tool in the field of functional genomics. By simply designing and introducing a dsRNA sequence that is complementary to a region of a target gene transcript, loss-of-function phenotypes can be generated quickly and easily.

C. Establishing RNAi in Mammalian Cells

A number of groups have designed plasmid expression vectors to generate sustained production of siRNAs by transient or stable transfection. Some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which are processed *in vivo* into siRNA-like molecules capable of carrying out gene-specific silencing (Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; and Yu *et al.*, 2002). After construction is complete, these vectors contain a DNA sequence that encodes the shRNA cloned between a Pol III promoter and a transcription termination site comprising 4–5 thymidine residues. The transcript is terminated at position 2 of the termination site and then folds into a stem-loop structure with 3' UU-overhangs (Figure 2). The ends of the shRNAs are processed *in vivo*, converting the shRNA into ~21 nt siRNA-like molecules, which in

I. Introduction *continued*

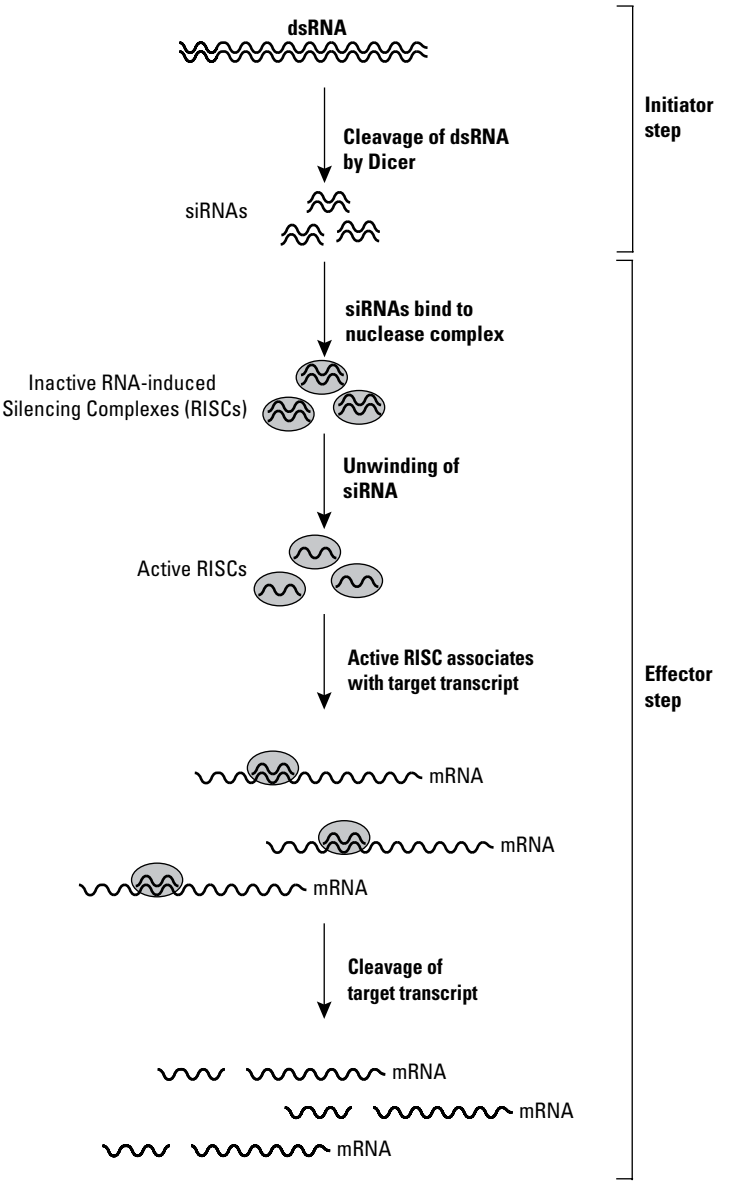


Figure 1. Mechanism of RNA interference (RNAi).

I. Introduction *continued*

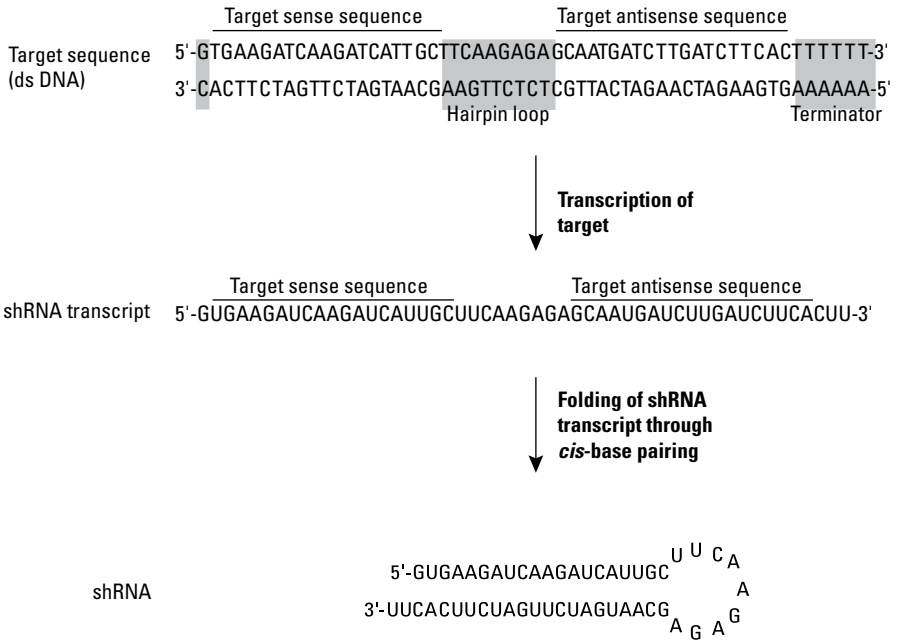


Figure 2. Small hairpin RNAs (shRNAs) generated using an oligonucleotide DNA sequence. This example shows a target sequence derived from the coding region of the ß-actin gene (Harborth *et al.*, 2001). This target sequence is cloned downstream of a Pol III promoter in an expression vector for gene silencing in mammalian cells. A hairpin loop sequence is located between the sense and antisense sequences on each complementary strand. The shRNA behaves as an siRNA-like molecule capable of carrying out gene-specific silencing. This mechanism is employed by all members of the pSIREN vector family.

turn initiate RNAi (Brummelkamp *et al.*, 2002). These vectors represent a definite improvement in initiating RNAi in cells; however not all cell types are easy to transfect using these vectors.

D. Overview of the Knockout™ Inducible RNAi System

The **Knockout™ Inducible RNAi Systems** combine tight on/off control of an shRNA with efficient and cost-effective shRNA delivery to many cell types. This system is a modified form of the tightly regulated, Tetracycline-controlled-gene expression system described by Gossen & Bujard (1992) and Gossen *et al.* (1995). The system is designed so that expression of an shRNA is induced when either tetracycline (Tc) or doxycycline (Dox; aTc derivative) is added to the culture medium of an appropriately engineered cell (Figure 3). Induction of the shRNA then results in suppression of the gene targeted by the shRNA through RNAi. Thus, the system allows for tight regulation of the expression of an shRNA—and the gene that the shRNA targets—in response to varying concentrations of Tc or Dox.

I. Introduction *continued*

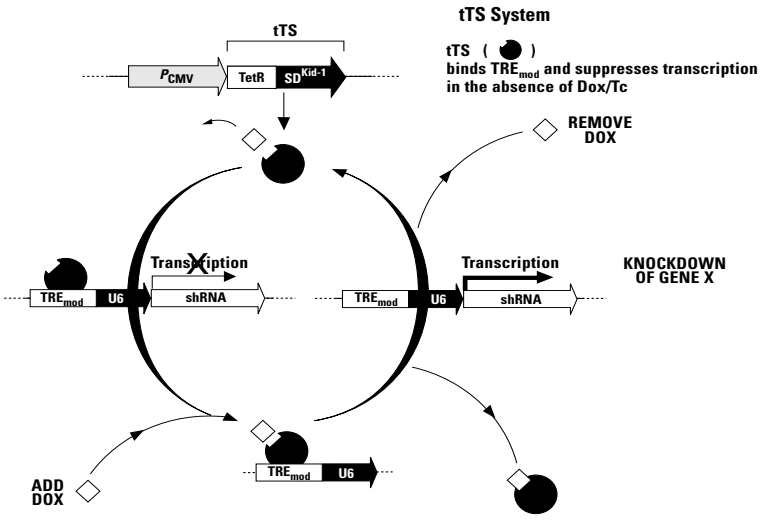


Figure 3. Schematic of gene regulation in the inducible tTS system. The suppressor tTS binds the *tetO* sequence in the TRE_{mod} promoter region of the response plasmid, suppressing shRNA expression. In the presence of Dox, the tTS dissociates from the TRE_{mod} and allows activation of shRNA transcription from the downstream U6 promoter. Please see Appendix and accompanying Vector Information Packets for maps and detailed vector information.

For this reason, this inducible RNAi system is well suited for instances where the constitutive suppression of a particular gene is extremely potent or toxic to the host cell.

As with standard Tet-systems used to control expression of mRNAs of interest, the Knockout Inducible RNAi Systems have two components: a **regulatory protein** and a **Tet-reponsive promoter**, the activity of which is regulated by binding of the regulatory component.

In the case of the Knockout Inducible RNAi Systems, the **regulatory protein** is the tetracycline-controlled transcriptional suppressor (**tTS**; April 1999 *Clontechniques*). tTS is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}), a powerful transcriptional suppressor (Freundlieb *et al.*, 1999; Witzgall *et al.,* 1994). By virtue of the TetR moiety, tTS can bind tightly Tet operator sequences (*tetO*) in the absence of Tc or Dox. The KRAB-AB domain then acts as a potent suppressor of transcription from any promoter downstream of the *tetO* sequences.

I. Introduction *continued*

The **response plasmid** in this system contains a Tet-responsive U6 promoter which is used to drive expression of the shRNA. This promoter ($P_{TREmod/U6}$) consists of the Tet response element (TRE)—seven direct repeats of the *tetO* 19-mer—upstream of a minimal U6 promoter. The TRE derives from the P_{tight} promoter used in the standard Tet-response plasmid pTRE-TIGHT (April 2002 *Clontechniques*).

In the absence of Dox, tTS binds the *tetO* sequence within the TRE and actively silences transcription of the shRNA from the minimal U6 promoter (Figure 3). As Dox is added to the culture medium, tTS dissociates from the TRE, relieving transcriptional suppression and allowing transcription to proceed from the hybrid TRE/U6 promoter.

The ultimate goal in setting up a functional and inducible RNAi system is to create a double-stable cell line that stably expresses both the regulatory and response plasmids. To accomplish this, you can either make use of the response and regulatory vectors provided in the kit to develop a cell line specific to your interest, or you can start from one of the premade Tet tTS stable regulatory cell lines (available separately) and introduce your shRNA into it under control of the response element. The two response vectors provided in this kit are the **RNAi-Ready pSIREN-RetroQ-TetH and pSIREN-RetroQ-TetP** vectors. These are inducible retroviral shRNA expression vectors. The vectors are provided linearized and ready for ligation with a dsDNA oligonucleotide encoding an shRNA. The pSIREN-RetroQ-TetH vector contains a hygromycin resistance gene for the selection of stable transformants, while the pSIREN-RetroQ-TetP vector contains a puromycin resistance gene. Both vectors are self-inactivating expression vectors designed to express the target shRNA without the risk of promoter interference from the upstream LTR in the integrated provirus (July 2002 *Clontechniques*, Julius *et al.*, 2000).

In addition to the response plasmids, the system provides two version of the regulator (tTS) construct. The first, ptTS-Neo, is a plasmid vector which can be used to transfect target cells of interest. The second, pQC-tTS-IN, is a retroviral vector that can be used to generate stable tTS-expressing cell lines by retroviral infection. Both plasmids contain the neomycin-resistance gene to allow for the end point selection of double stable cell lines containing both the tTS and your recombinant pSIREN-RetroQ-Tet-shRNA vector.

Both pSIREN-RetroQ-Tet constructs can either be transfected directly into target cells as plasmids or be used to generate virus by transfection into a suitable packaging line. The virus produced can then be used to infect a broad range of mitotically active target cells of interest.

I. Introduction *continued*

The retroviral vector is optimized to eliminate promoter inferference through self-inactivation.The hybrid 5' LTR of both vectors consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter.This construct drives high levels of transcription in HEK 293-based packaging cell lines that peaks 48 hr after transfection. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral transcript in the infected cell, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR (CMV) protomer.

Although pSIREN-RetroQ-Tet constructs can be introduced by either transfection or infection, we recommend the latter in order to avoid promoter interference issues described above (Barton *et al.*, 2002; Emerman *et al.*, 1984). Additionally, retroviral infection allows you to introduce your shRNA into virtually any mitotically active cell with high efficiency. A detailed discussion of the advantages of retroviral delivery of RNAi constructs can be found in Section I of the Retroviral Gene Transfer and Expression User Manual (PT3132-1). The Retroviral User Manual also provides protocols for packaging recombinant pSIREN-RetroQ-Tet constructs into infectious, replication-incompetent particles. Additional information can be found on our Retroviral Gene Expression Resource at **www.clontech.com/expression/retro/index.shtml.**

Furthermore, each RNAi system contains a control pSIREN-RetroQ-Tet-Luc vector for silencing luciferase gene expression when tested in cells expressing luciferase from a suitable vector. This control vector can be used to quickly and easily monitor the consecutive steps leading to the generation of a double-stable inducible RNAi line because it is a highly effective knockdown regulatory construct that has been quantitatively validated.

This User Manual provides protocols for generating your own inducible RNAi system through shRNA oligonucleotide sequence design, annealing of shRNA oligonucleotides, ligation of annealed oligonucleotides into pSIREN-RetroQ-Tet vectors, delivery of pSIREN-RetroQ-Tet-shRNA constructs into cell lines, and establishing stable Tet tTS and double-stable inducible RNAi cell lines. Extensive online tools to assist you with shRNA oligo sequence design can be found at **http://bioinfo2.clontech.com/rnaidesigner/**.

I. Introduction *continued*

E. Advantages of the Knockout™ RNAi Inducible Systems

The Knockout Inducible RNAi Systems have several advantages over other regulated gene expression systems that function in mammalian cells:

- Extremely tight on/off regulation. Background or leaky expression of shRNA in the absence of induction is extremely low with constructs such as pSIREN-RetroQ-Tet vectors. With any inducible RNAi system, tight regulation is crucial because even low levels of leaky shRNA expression can result in significant suppression of the target gene.
- Fast response times and high levels of induction. With our Inducible RNAi system, knockdown of a target can be seen within 24 hrs of Dox addition. Typically, maximal knockdown is seen by 48 hrs post Dox addition. In contrast, other systems for inducible expression exhibit slow induction (up to several days) and, in transient transfections, require pretreatment with Dox for 1–2 days prior to transfection to ensure that repression is fully alleviated. This can result in incomplete induction (compared to repressor-free controls). Additionally, such repressor-based systems may require high amounts of Dox (2–5 µg/ml) to ensure full removal of the repressor. We have found the tTS-based system to be sensitive to as little as 10 ng/ml Dox (see Figure 6).
- Other inducible RNAi systems rely solely on direct steric-hindrance of polymerase binding, through the action of repressor proteins, such as TetR alone. Such repression-based systems require very high—and difficult to attain—levels of repressor to ensure 100% occupancy of the regulatory sites. Even if suitably high levels of repressor can be obtained, the presence of high repressor levels makes it difficult to achieve rapid, high-level induction (Yao *et al.,* 1998). In the system described here, regulation of the promoter is an active process, controlled by the action of a transcriptional suppressor, tTS. In contrast to other regulatory systems for inducible expression of shRNAs, the tTS protein actively suppresses polymerase activity at the promoter rather than simply blocking the binding of the polymerase to the TATA box in a passive manner. Thus, the system does not require such high-level expression of the suppressor, making induction faster and more tightly- controlled.

II. List of Components

Store all components at –20°C.

Visit our Tet Systems product page at **www.clontech.com** for a current list of cell lines and products available for the Tet Systems.

The following reagents are sufficient for 40 ligations into either the pSIREN-RetroQ-TetH or pSIREN-RetroQ-TetP vectors.

Knockout™ Tet RNAi System H (Cat. No. 630925)

- 2 µg RNAi-Ready pSIREN-RetroQ-TetH Vector (linearized, 50 ng/µl)
- 20 µg ptTS-Neo Vector (500 ng/µl)
- 20 µg pQC-tTS-IN Vector (500 ng/µl)
- 20 µg pSIREN-RetroQ-TetH-Luc Vector (500 ng/µl)
- 50 ml Tet System Approved Fetal Bovine Serum
- RNAi-Ready pSIREN-RetroQ-TetH Vector Information Packet (PT3811-5)
- ptTS-Neo Vector Information Packet (PT3813-5)
- pQC-tTS-IN Vector Information Packet (PT3822-5)

Knockout™ Tet RNAi System P (Cat. No. 630926)

- 2 µg RNAi-Ready pSIREN-RetroQ-TetP Vector (linearized, 50 ng/µl)
- 20 µg ptTS-Neo Vector (500 ng/µl)
- 20 µg pQC-tTS-IN Vector (500 ng/µl)
- 20 µg pSIREN-RetroQ-TetP-Luc Vector (500 ng/µl)
- 50 ml Tet System Approved Fetal Bovine Serum
- RNAi-Ready pSIREN-RetroQ-TetP Vector Information Packet (PT3812-5)
- ptTS-Neo Vector Information Packet (PT3813-5)
- pQC-tTS-IN Vector Information Packet (PT3822-5)

III. Additional Materials Required

The following materials are required but not supplied:

For cloning of shRNA oligonucleotides into pSIREN-RetroQ-Tet vectors

- **T4 DNA ligase** (New England Biolabs, Cat. No. M0202S). 10X T4 DNA Ligase Buffer is provided with the enzyme.
- **Bovine serum albumin (BSA),** 10 mg/ml
- **Nuclease-free H_2O**
- **Fusion-Blue™ Competent Cells** (Cat. No. 636700)
- **NucleoSpin® Multi-8 Plus Plasmid Kit** (Cat. No. 635976)
- **NucleoBond® Plasmid Maxi EF Kit** (Cat. No. 635953)

For cell culture

- **Tissue culture plates and flasks,** available from BD Discovery – Labware (**www.bdbiosciences.com/discovery_labware**)
- **Dulbecco's Modified Eagle's Medium** (DMEM; Sigma, Cat. No. D5796), **Alpha Minimal Essential Medium Eagle** (alpha-MEM), **RPMI-1640**, or other specified medium.
- **Fetal bovine serum (FBS)**. It is critical that the FBS in your tissue culture media not inhibit Tet-responsive expression. **You can eliminate Tc contamination problems by using the Clontech's Tet System Approved FBS that has been functionally tested in the Tet Systems to ensure against possible Tc contamination.** Each Inducible RNAi System includes 50 ml of Tet System Approved FBS. Additional FBS is available for purchase in a wide variety of sizes (Cat. Nos. 631105, 631101, 631107 & 631106). For more details about FBS and Tc contamination, please see Section VII.A of the Tet-Off® and Tet-On® Gene Expression Systems User Manual (PT3001-1).
- **200 mM L-Glutamine** (Sigma Cat. No. G7513)
- Solution of 10,000 units/ml **Penicillin G sodium** and 10,000 µg/ml **Streptomycin sulfate** (Sigma Cat. No. P0781)
- **Trypsin/EDTA** (VWR/Hyclone Cat. No. 16777-166)

III. Additional Materials Required *continued*

- **2X HEPES buffered saline** (2X HBS) (pH 7.1)

	Final Conc.	To prepare 100 ml
Na_2HPO_4	1.5 mM	0.018 g
NaCl	280 mM	1.64 g
HEPES	50 mM	1.19 g

Dissolve components in 100 ml of distilled H_2O. Adjust to pH 7.1 with 0.5 N NaOH. Pass through 0.22-micron filter. Store in 5 ml aliqouts at -20°C.

- **Phosphate buffered saline** (PBS) (pH 7.4)

	Final Conc.	To prepare 2 L
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve components in 1.8 L of distilled H_2O. Adjust to pH 7.4 with 0.1 N NaOH. Add ddH_2O to final volume of 2 L. Autoclave. Store at room temp.

- **Antibiotics for clonal selection**

  Prior to use, determine the optimal concentration of each antibiotic for selection as described in Section VIII.A.

  - **G418** (for selection of tTS stable cell lines)

    G418 is available in powdered form from Clontech (Cat. No. 631307). Note that the effective weight is about 0.7 g per gram of powder. Make a 10 mg/ml stock solution by dissolving 1 g of powder in approximately 70 ml of DMEM or alpha-MEM (without supplements). Filter sterilize and store at 4°C.

    Recommended working concentration:
    Maintenance: 100 µg/ml
    Selection (HeLa or CHO cells): 400–500 µg/ml
    (acceptable range): 50–800 µg/ml

  - **Hygromycin** (for selection of pSIREN-RetroQ-TetH Cell Lines)

    Hygromycin is available from Clontech (Cat. No. 631309).

    Recommended working concentration:
    Maintenance: 100 µg/ml
    Selection (HeLa or CHO cells): 200 µg/ml
    (acceptable range): 50–800 µg/ml

  - **Puromycin** (for selection of pSIREN-RetroQ-TetP Cell Lines)

    Puromycin is available from Clontech (Cat. Nos. 631305 & 631306)

    Recommended working concentration:
    Maintenance: 0.5 µg/ml
    Selection (acceptable range): 0.5–5 µg/ml

III. Additional Materials Required *continued*

- **Cell Freezing Medium, with or without DMSO** (Sigma Cat. Nos. C6164 & C6039)
- **Cloning cylinders** (PGC Scientific Cat. Nos. 62-6150-40, 62-6150-45, 62-6151-12 & 62-6151-16)
- **Antibiotics for Tet Induction**
  - **Doxycycline** (Cat. No. 631311). Dilute to 1–2 mg/ml in H_2O. Filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.
  - **Tetracycline hydrochloride** (Sigma Cat. No. T3383). Dilute to 1–2 mg/ ml in 70% ethanol. Filter sterilize, aliquot, and store at –20°C in the dark. Use within two months.

For delivery of pSIREN-RetroQ-Tet and tTS vectors

- **Clonfectin™ Transfection Reagent** (Cat. No. 631301)
- **CalPhos™ Mammalian Transfection Kit** (Cat. No. 631312)
- **Retro-X™ Universal Packaging System** (Cat. No. 631530)

For luciferase assays

- **Luciferase expression vector.** We recommend the pGL2 Control Vector from Promega (Cat. No. E1611).
- **Luciferase Reporter Assay Kit** (Cat. No. 631714)

IV. Protocol Overview

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Figure 4 shows an overview of the procedure described in this User Manual, which provides protocols for shRNA oligonucleotide sequence design, annealing of shRNA oligonucleotides, ligation of annealed oligonucleotides into pSIREN-RetroQ-Tet vectors, delivery of these constructs (with transfection or infection) into target cells, and establishing double-stable inducible RNAi cell lines. Extensive online tools to assist you with shRNA oligonucleotide sequence design can be found at **http://bioinfo2.clontech.com/rnaidesigner/**.

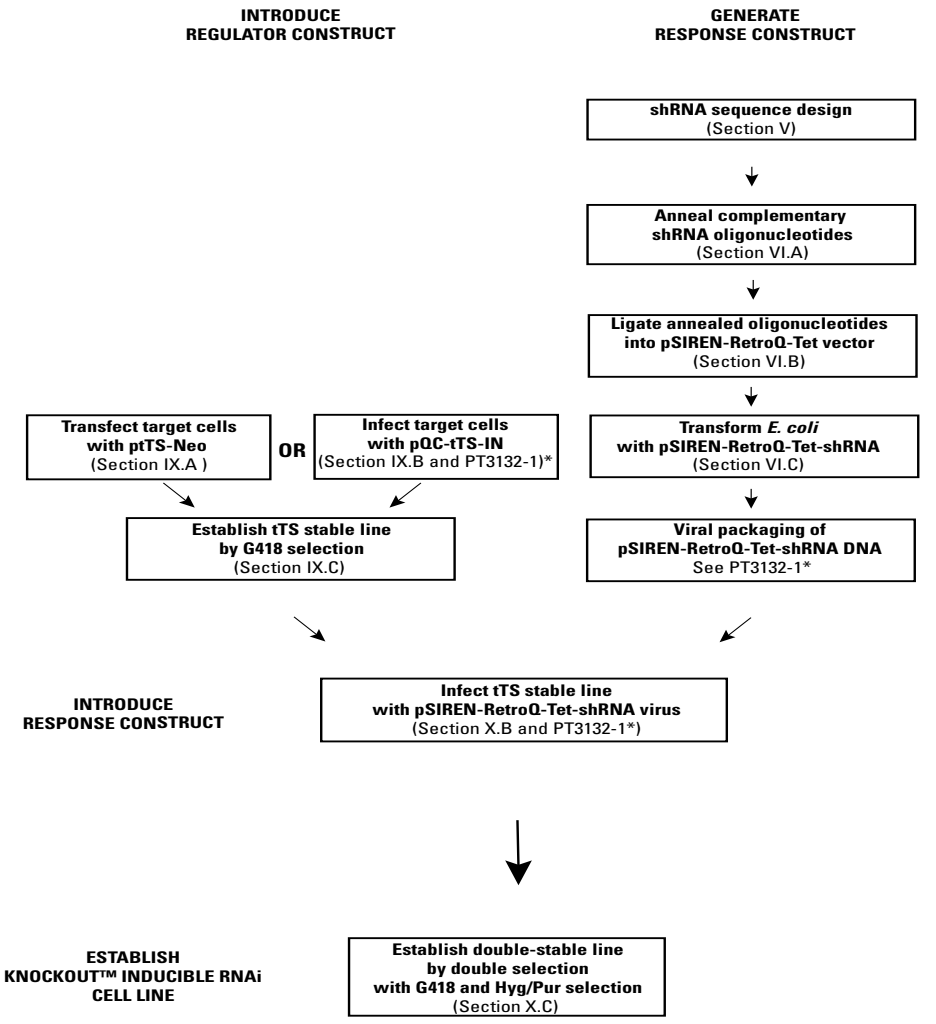
shRNA Oligonucleotide Design (Section V)

- The success of your experiment depends on choosing the proper target sequence within your gene of interest and the proper design of the shRNA oligonucleotides. In addition, we highly recommend that you test more than one shRNA sequence per gene of interest.
- PAGE purification of your designed oligonucleotides ensures that a higher percentage of the oligonucleotides will be full-length and therefore increases the chance of cloning a complete and functional insert. When using PAGE-purified oligonucleotides, we typically achieve 80–90% of clones with the correct insert.
- When testing your pSIREN-RetroQ-Tet construct for functionality, you will need a gene-specific assay to test for the suppression of Gene X.

Examples of assays that can be used include:

- Western blot with an antibody to Protein X
- RT-PCR using Gene X primers. Be sure you can discriminate between PCR products generated from true RT-PCR products and those from genomic DNA.
- Northern blot with specific probe to Gene X
- Functional assay for Protein X
- ProLabel Screening Kits. Our screening kits allow fast and quantititative chemiluminescent measurement of expression levels of any gene fused to the ProLabel tag. The kits are supplied in two formats, a Creator™ format for genes already cloned into the Creator backbone (Cat. No. 631542) and an In-Fusion™ format for PCR cloning of precise, direction al constructs (Cat. No. 631724). For more details, please see the ProLabel Screening Kit User Manual (PT3789-1).

IV. Protocol Overview *continued*



*Retroviral Gene Transfer and Expression Systems User Manual (PT3132-1)

Figure 4. Overview of the Knockout™ Inducible RNAi Systems procedure. This procedure illustrates the primary steps to generate an inducible RNAi cell line using two consecutive introductions of recombinant DNA–the regulator construct and the response construct.

IV. Protocol Overview *continued*

Plasmid DNA purity for transfections (Section VI)

To ensure DNA purity, isolate all plasmids for transfection using a NucleoBond Plasmid Maxi EF Kit (Cat. No. 635953) or by CsCl density gradient purification (Sambrook *et al.*, 2001).

Important note on simultaneous versus consecutive transfections

In general, we recommend that you do not attempt to save time by cointroducing, using either transfection or viral delivery methods, the regulator and response plasmids. Cotransfected plasmids tend to cointegrate into the chromosome, and enhancer elements from the CMV promoter on the regulator construct can induce basal expression of the TRE/U6 promoter on the response construct. Furthermore, cotransfection prevents comparison of multiple clones, since differences in induction or absolute expression could be due to clone-to-clone variation in expression of tTS and/or pSIREN-RetroQ-Tet vectors.

In contrast, consecutive introduction of the regulator and response plasmids has several advantages. Most importantly, the response plasmid generally will not cointegrate with the regulator, allowing you to select a double-stable inducible RNAi cell line that gives very low to no background expression of your shRNA. Furthermore, once you have developed a suitable inducible RNAi cell line, it provides a proven genetic background into which you can introduce many different response plasmids.

V. shRNA Oligonucleotide Design

This section describes the process of identifying target sequences within a gene of interest and designing the corresponding oligonucleotides to generate the shRNA. Comprehensive online tools to assist you with shRNA oligonucleotide design can be found at **http://bioinfo2.clontech.com/rnaidesigner/**.

A. Selecting Target Sequences

1. Choose a region of 19 nucleotides. Do not select sequences within the 5' and 3' untranslated regions (UTRs) nor regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites (Elbashir e*t al.*, 2001). UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.

   Do not select sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination the shRNA transcript.
2. Calculate the GC content of the selected 19-base oligonucleotide sequence. The GC content should be between 40% and 60%; a GC content of approximately 45% is ideal.
3. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence also appear to have increased knockdown activity.
4. Check the 19-base oligonucleotide for secondary structure and long base runs, both of which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
5. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and show no significant homology to other genes. Candidate sequences that meet these criteria are potential shRNA target sites.

   To optimize gene silencing, we highly recommend that you test more than one shRNA target sequence per gene. We provide enough pSIREN-RetroQ-Tet vector to perform 40 ligations, which allows you to screen for functional shRNA sequences within your gene of interest. You should test at least 4 shRNAs per gene. It may help to choose shRNA targets that are distributed along the length of the gene sequence to reduce the chance of targeting a region that is either highly structured or bound by regulatory proteins.

   Note: You will need to design a gene-specific assay to test for the suppression of Gene X, if you have not already done so. See Section IV for additional information.

B. Designing Oligonucleotides

It is necessary to synthesize two complementary oligonucleotides (a top strand and a bottom strand) for each shRNA target site. Figure 5 illustrates the overall structure of the prototypical oligonucleotide

V. shRNA Oligonucleotide Design *continued*

sequences for use in pSIREN-RetroQ-Tet vectors. The sequences of the oligonucleotides should include:

- A 5'-*Bam*H I restriction site overhang on the top strand and a 5'-*Eco*R I restriction site overhang on the bottom strand. These restriction sites will enable directional cloning of the annealed oligonucleotides into the pSIREN-RetroQ-Tet vector.
- A guanine (G) residue added upstream of the 5'-end of the shRNA sense strand, if the target sequence does not start with a purine (preferred as Pol III transcription start site).
- The 19-base oligonucleotide sense sequence (target sense sequence) of the shRNA target site. **Important:** This should not contain a consecutive run of 3 or more thymidine residues, which can potentially cause premature termination of transcription.
- A 7–9 nucleotide hairpin loop sequence. (We typically use 5'-TTCAAGAGA- 3'; see Sui *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Brummelkamp *et al.*, 2002; and Paul *et al.*, 2002 for other effective loop sequences.)
- The 19-base oligonucleotide antisense sequence (target antisense sequence) of the shRNA target site; ensure proper orientation for correct formation of the hairpin structure (see Figure 2). **Important:** The target antisense sequence should not contain a consecutive run of 3 or more thymidine residues, which can potentially cause premature termination of transcription.
- A RNA Pol III terminator sequence consisting of a 5–6 nucleotide poly(T) tract.
- [Optional, but recommended] A unique restriction site immediately downstream of the terminator sequence for restriction digest analysis to confirm the presence of the cloned insert

A typical oligonucleotide has 5 bases for the restriction site at the 5' end (when digested with *Bam*H I), 19 bases of sense strand, 7–9 bases of hairpin loop, 19 bases of antisense strand, 6 bases of terminator, 6 bases of a unique restriction site, and 1 base for the restriction site at the 3' end (when digested with *Eco*R I)—resulting in an oligonucleotide of 63–65 bases. See Table I for examples of sense and antisense sequences designed for selected genes. Our comprehensive online designer tool at **http://bioinfo2.clontech.com/rnaidesigner/** can design the required oligonucleotides for any sequence inputted.

See Section IV for our recommendation to use PAGE-purified oligonucleotides. It is possible to clone without PAGE purification, but it is likely that the overall ligation efficiency and the number of correct clones will decrease due to the presence of incomplete oligonucleotide extensions. If the oligonucleotides are PAGE-

V. shRNA Oligonucleotide Design *continued*

purified, order them at the 200 nmol scale. There is no need to order phosphorylated oligonucleotides. pSIREN-RetroQ-Tet Vectors have not been dephosphorylated after linearization; thus ligation will proceed smoothly using unphosphorylated oligonucleotides. In fact, use of phosphorylated oligonucleotides may reduce your cloning efficiency.

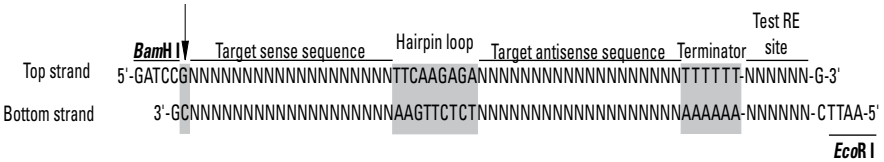


Figure 5. shRNA oligonucleotide sequence design. The arrow denotes the purine residue required for RNA Pol III to initiate transcription. The hairpin loop sequence shown is one of many functional loop sequences used to generate shRNAs. Termination is signaled using a poly(T) tract. Including a unique restriction site (Test RE site) allows confirmation of the cloned insert after the ligation and transformation reactions. 5' *Bam*H I and 3' *Eco*R I overhangs are necessary for directional cloning into pSIREN-RetroQ-Tet vectors. See Table I for examples of target sense and antisense sequences for selected genes.

TABLE I. EXAMPLES OF PUBLISHED TARGET SEQUENCES[a]

Gene	Target sequence[b]	Sense sequence	Antisense sequence	Reference
β-actin	AATGAAGATCAAGATCATTGC	TGAAGATCAAGATCATTGC	GCAATGATCTTGATCTTCA	Harborth *et al.*, 2001
Bcr-abl	AAGCAGAGTTCAAAGCCCTT	GCAGAGTTCAAAGCCCTT	AAGGGCTTTGAACTCTGC	Scherr *et al.*, 2002
hRad9	AAGTCTTTCCTGTCTGTCTTT	GTCTTTCCTGTCTGTCTTT	AAAGACAGACAGGAAAGAC	Hirai & Wang, 2002

[a] Sequences are shown for top strand oligo design. All sequences shown 5' to 3'. Bottom strand oligo design (not shown) is the complementary sequence to the top strand.
[b] Identified from gene coding sequence.

VI. Cloning into pSIREN-RetroQ-Tet Vectors

A. Annealing the Oligonucleotides

For convenience, Steps 3–6 can be performed in a thermal cycler.

1. Resuspend each purified oligonucleotide in TE buffer to a final concentration of 100 µM.
2. Mix the oligos for the top strand and the bottom strand at a 1:1 ratio. This mixture will ultimately yield 50 µM of ds oligo (assuming 100% theoretical annealing).
3. Heat the mixture to 95°C for 30 sec to remove all secondary structure.

   Note: Heating to 95°C ensures that the internal hairpin of each oligonucleotide is disrupted and promotes intermolecular annealing.
4. Heat at 72°C for 2 min.
5. Heat at 37°C for 2 min.
6. Heat at 25°C for 2 min.
7. Store on ice.

The annealed oligonucleotide is now ready for ligation into the pSIREN-RetroQ-Tet vector. Alternatively, the annealed oligonucleotide can be stored at –20°C until ready to use.

B. Ligating the ds Oligonucleotide Into pSIREN-RetroQ-Tet Vectors

1. Dilute the annealed oligo (from Step A.7) with TE buffer to obtain a concentration of 0.5 µM.

   Note: To ensure good ligation efficiency it is necessary to dilute the oligo so that it is only in moderate excess. Using an excess of the oligo will inhibit ligation.
2. Assemble a ligation reaction for each experimental annealed oligonucleotide. For each ligation, combine the following reagents in an Eppendorf tube:

1	µl	RNAi-Ready pSIREN-RetroQ-Tet Vector (50 ng/µl)
1	µl	Diluted, annealed oligonucleotide (0.5 µM)
1.5	µl	10X T4 DNA ligase buffer
0.5	µl	BSA (10 mg/ml)
10.5	µl	Nuclease-free H_2O
0.5	µl	T4 DNA ligase (400 U/µl)
15	µl	Total volume

   Note: If desired, a control ligation can be assembled using 1 µl of nuclease-free H_2O instead of annealed oligonucleotide.
3. Incubate the reaction mixture for 3 hr at room temperature.

   Note: Do not let the ligation reaction go longer than 3 hr. If you are unable to immediately perform the transformation after this step, store the completed ligation at –20°C until ready to use.

VI. Cloning into pSIREN-RetroQ-Tet Vectors *continued*

C. Transforming Fusion-Blue™ Competent Cells with Recombinant pSIREN-RetroQ-Tet Vectors

Fusion-Blue Competent Cells are an *E.coli* K-12 strain that provides high transformation efficiency. The strain carries *rec*A and *end*A mutations that make it a good host for obtaining high yields of plasmid DNA. We routinely use this strain for our shRNA cloning.

1. Thaw the required number of tubes of cells on ice for 10 min. Tap gently to ensure that the cells are suspended.
2. Add 2 µl of the ligation mixture (from Step B.3) directly to 50 µl of cell suspension. Mix gently to ensure even distribution of the DNA solution.
3. Incubate the transformation mixture (DNA + cells) on ice for 30 min.
4. Heat the tubes for precisely 45 sec in a water bath at 42°C without shaking.
5. Remove the tubes from the water bath and place them directly on ice for 1–2 min.
6. Add 950 µl room-temperature SOC medium to each tube. Incubate at 37°C for 60 min while shaking at 250 rpm.
7. Plate 20–150 µl from each transformation on selective medium containing the appropriate concentration of antibiotic. Incubate at 37°C.

   Notes

   - Both cell competency and ligation efficiency affect the outcome of the transformation. We suggest plating different amounts on separate plates to identify the optimal volume for determining transformation efficiency and isolating colonies.
   - Plating is accomplished by spreading cells on selective medium [e.g., LB agar + Ampicillin (50–100 µg/ml)]. Please see the Vector Information Packet that accompanies the pSIREN-RetroQ-Tet Vector for details.
8. Inoculate a small-scale liquid culture with a single, well-isolated colony. We recommend you set up 4–8 such cultures to ensure you obtain at least one positive clone. After overnight incubation, isolate plasmid DNA using any standard method. For small-scale purification (≤ 20 µg plasmid DNA), we recommend our NucleoSpin Plasmid Kit (Cat. No. 635987).
9. Identify the desired recombinant plasmid by restriction analysis using the unique restriction site within the shRNA oligonucleotide sequence. If desired, verify your insert by sequencing.

   Note: Since there is always a chance for mutations in the oligo due to synthesis errors, we strongly recommend that you sequence at least two clones to verify the correct oligo sequence. Because hairpin sequences are difficult to sequence, inform your sequencing facility so that sequencing conditions can be adjusted accordingly.

VI. Cloning into pSIREN-RetroQ-Tet Vectors *continued*

10. Once a positive clone has been identified, inoculate a large-scale liquid culture to prepare greater quantities of recombinant pSIREN-RetroQ-Tet-shRNA vector. To ensure optimal purity of the DNA, isolate all plasmids for transfection using a NucleoBond Plasmid Maxi EF Kit (Cat. No. 635953) or by CsCl density gradient purification (Sambrook *et al.*, 2001).

VII. Cell Culture Guidelines

A. General Information

The protocols in this User Manual provide only general guidelines for mammalian cell culture techniques. Perform all steps involving cell culture using sterile technique in a suitable hood. For those requiring more information on mammalian cell culture, we recommend the following general references:

- *Culture of Animal Cells,* Fourth Edition, ed. by R. I. Freshney (2000, Wiley-Liss, NY)
- *Current Protocols in Molecular Biology*, ed. by F. M. Ausubel *et al.* (2003, Wiley & Sons)

B. Tetracycline vs. Doxycycline

The Knockout Inducible RNAi system responds equally well to either tetracycline (Tc) or doxycycline (Dox). However, we recommend that you use Dox, in part because a significantly lower concentration of Dox is required for complete activation (0.01–1 µg/ml Dox vs. 1–2 µg/ml Tc). In addition, Dox has a longer half-life (24 hr) than Tc (12 hr). Either antibiotic is used at concentrations far below cytotoxic levels seen for cell culture and transgenic studies. Other Tc derivatives have been used successfully as the inducer in Tet systems (Gossen & Bujard, 1993). See Section I.E. of the Tet-Off® and Tet-On® Gene Expression Systems User Manual (PT3001-1) for further discussion.

C. Characteristics of Premade Tet Cell Lines

General cell culture conditions: Premade Tet Cell Lines should be grown at 37°C in a humidified chamber with 5–10% CO_2. See the PAC for details particular to each cell line.

Relative growth rates: The incubation times in this User Manual are for cells such as CHO or HeLa with relatively rapid doubling times. Other cell types will differ in their growth rates.

Selection in G418 and hygromycin or puromycin: Maintain stable Tet tTS and double-stable inducible RNAi cell lines in the appropriate selective medium; however, the concentration of drug required for maintenance can be reduced from the levels used to select stably transfected clones. See Section III for suggested antibiotic concentrations. You may wish to alternate between selecting and nonselecting conditions for optimal results.

VII. Cell Culture Guidelines *continued*

D. Starting Tet Cell Cultures From Frozen Stocks

Note: Frozen cells should be cultured immediately upon receipt or as soon thereafter as possible. Increased loss of viability may occur after shipping if culturing is delayed.

1. Thaw vial of cells rapidly in a 37°C water bath with constant agitation. Immediately upon thawing, wipe the outside of the vial with 70% EtOH. Transfer the contents of the vial to a 10 cm dish, T25, or T75 flask, containing 1 ml of medium (without antibiotics). Mix gently.
2. Add an additional 4 ml of medium to the flask/dish and mix gently.
3. Add additional medium to the culture as follows:
   T25 flask or 10 cm dish add 5 ml (for a total volume of 10 ml)
   T75 flask add 10 ml (for a total volume of 15 ml)
4. Mix the cell suspension thoroughly. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place it in a 37°C humidified incubator (5–10% CO_2 as appropriate).
5. [Alternative method] The cells can also be rinsed prior to incubation. If rinsing is desired, perform Steps 1 and 2 in a 15 ml conical centrifuge tube. Centrifuge at 125 x g for 10 min, and resuspend in complete medium for culturing. This step removes the cryopreservative and can be beneficial when resuspending in small volumes. However, this step can damage fragile cell membranes.
6. The next day, examine the cells under a microscope. If the cells were not rinsed upon thawing (Step 5), centrifuge cells (if suspension cultures), aspirate the medium, and replace with fresh, prewarmed, complete medium (without antibiotics).
7. Expand the culture as needed. Note: The appropriate selective antibiotic(s) may be added to the medium after 48–72 hr in culture.

E. Preparing Frozen Stocks of Inducible RNAi Cell Lines

Once you have started growing aTet system cell line—either a premade one from Clontech or one of your own cell lines—prepare frozen aliquots to ensure a renewable source of cells.

1. Trypsinize the desired number of flasks.
2. Pool cell suspensions together, count cells, and calculate total viable cell number.
3. Centrifuge cells at 125 x g for 10 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x10^6 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or freeze cells in 70–90% FBS, 0–20% medium (no additives), and 10% DMSO.

VII. Cell Culture Guidelines *continued*

5. Dispense 1 ml aliquots into sterile cryovials.
6. Freeze slowly (1°C per min). Nalgene makes cryo-containers (Nalgene Cat. No. 5100) for this purpose if a specialized freezer is not available (freeze at –80°C overnight). Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C overnight. Remove vials from styrofoam container or cryo-containers the following day and place in liquid nitrogen or ultralow-temperature freezer (–150°C) for storage.
7. (Two or more weeks later) Plate a vial of frozen cells to confirm viability.

VIII. Pilot Experiments

A. Titrating G418, Hygromycin, and Puromycin (Kill Curves)

Prior to using G418, hygromycin, or puromycin to establish stable and double-stable cell lines, it is important to titrate your selection agent stocks to determine the optimal concentration for selection with the particular host cell line being tested. This is also important because of lot-to-lot variation in the potency of these drugs. Therefore, you should titrate each new lot of antibiotic to determine the optimal concentration. We recommend that you perform two experiments for each drug: (1) a titration to determine the optimal drug concentration, and (2) an experiment to determine the optimal plating density. This step is recommended even if you are using one of our premade Tet tTS cell lines (see Related Products).

1. Titrate at fixed cell density.
   a. Plate 2 x 10^5 cells in each of six 10 cm tissue culture dishes containing 10 ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, 800 µg/ml) of hygromycin or G418. For puromycin, add the drug at 0, 0.5, 1, 1.5, 3, and 6 µg/ml.

      Note: Our premade HEK 293 Tet tTS cell line is especially sensitive to hygromycin; test a concentration range with a midpoint of 25 µg/ml.

   b. Incubate the cells for 10–14 days, replacing the selective medium every 4 days (or more often if necessary).
   c. Examine the dishes for viable cells every 2 days.

   For selecting stable transformants, use the lowest concentration that begins to give massive cell death in ~5 days and kills all the cells within two weeks. For HeLa and CHO cells, we have found 400 µg/ml G418 and 200 µg/ml hygromycin to be optimal. In mammalian cells the optimal level of puromycin is typically around 1 µg/ml.
2. Determine optimal plating density.

   Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluency before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, large cells that double rapidly have a lower optimal plating density than small cells that double slowly.
   a. Plate cells at several different densities in each of six 10 cm tissue culture dishes containing 10 ml of the appropriate selective medium. Suggested densities (cells/10 cm dish): 5 x 10^6, 1 x 10^6, 5 x 10^5, 2 x 10^5, 1 x 10^5, and 5 x 10^4.
   b. Incubate the cells for 5–14 days, replacing the selective medium every 4 days.

VIII. Pilot Experiments *continued*

c. Examine the dishes for viable cells every 2 days.

For selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluency before massive cell death begins (at about day 5). This is the cell density at which cells should be plated for selection of stable transfectants. For example, in HeLa cells we have found 2 x 10^5 cells/10 cm dish to be a good plating density.

B. [Recommended] Test Potential Host Cells By Transient Transfection with ptTS-Neo and pSIREN-RetroQ-Tet-Luc

Tet expression systems have been established in numerous cell lines including HeLa, MCF7, and HEK 293. However, the system may not be compatible with every cell type. Performing a transient expression assay with ptTS-Neo, pSIREN-RetroQ-Tet-Luc, and a luciferase expression vector may provide a quick indication of whether or not the Inducible RNAi system will work in a particular cell line. This pilot experiment is not necessary if you are working with a premade Tet tTS cell line because it has already undergone testing and validation with our Knockout Inducible RNAi System.

You should transfect cells using varying ratios of ptTS-Neo, the appropriate pSIREN-RetroQ-Tet-Luc control vector, and a luciferase vector. As a starting point you can try the following ratio of the constructs:

ptTS-Neo	:pSIREN-RetroQ-Tet-Luc	:luciferase vector
2 µg	: 1 µg	: 1 µg
2 µg	: 2 µg	: 1 µg

When using HEK 293 cells plated at 1 x 10^5 per well (roughly 60-70% confluent) and transfected with the 2:1:1 ratio of ptTS-Neo:pSIREN-RetroQ-TetP-Luc:pCMV-Luc, we consistently observe a 60-75% knockdown of luciferase activity with 48 hr of 1 µg/ml Dox induction as shown in Figure 6. When using MCF7 or HeLa cells, we observe a 60-75% knockdown of Luciferase activity within 72 hr of 1 µg/ml Dox induction.

Important Note: Knockdown levels are almost always lower in transient assays than in properly screened stable and double-stable cell lines since the amount of plasmid in the cell may titrate out the tTS to some degree. Therefore, an apparent lack of induction response in the transient assay should not be the sole reason for aborting your experiments in a particular cell line. In this case titration of the input plasmids may help to give better induction profiles.

VIII. Pilot Experiments *continued*

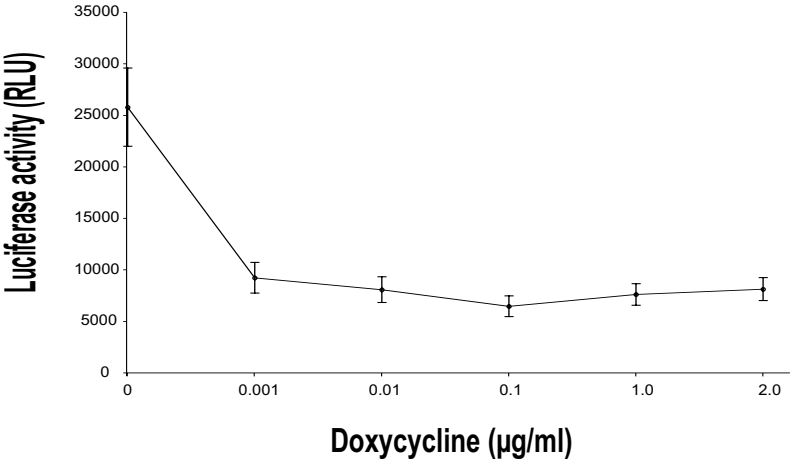


Figure 6. Dox dose-response curve for analyzing knockdown by transient transfection. HEK 293 cells plated at 1 x 10^5 per well were transiently transfected with ptTS-Neo, pSIREN-RetroQ-TetP-Luc, and pCMV-Luc in a 2:1:1 ratio. A dose response curve exhibits a 75% knockdown in relative light units (RLU) of luciferase activity with 48 hr of 1 µg/ml Dox induction. Similar knockdown results were obtained with HeLa cells (data not shown).

IX. Development of Tet tTS Stable Cell Lines

Skip Section IX if you have purchased a premade Tet tTS Cell Line

The following protocols describe the development of Tet cell lines stably expressing the tTS regulator plasmid. You must optimize the protocol for each cell type. The parameters most likely to need adjustment include plating densities, delivery method, G418 concentrations for selection, and incubation and growing times. The tTS regulatory protein can be delivered by either transfection or infection methods. Introduction by transfection is accomplished with the plasmid vector (ptTS-Neo) as outlined in Step A. If you are delivering the tTS regulatory construct via infection, this method requires the retroviral vector (pQC-tTS-IN) and is outlined in Step B.

Regardless of the cell type and delivery method, the goal is to generate a Tet cell line that gives both high expression of luciferase activity in the absence of Dox, and high knockdown of luciferase activity in the presence of Dox. These qualities can be tested by transient cotransfection with a luciferase expression vector and pSIREN-RetroQ-Tet-Luc as described in Section IX.C. Because the level of expression of tTS is profoundly affected by the site of integration, we recommend that you isolate and analyze as many clones as possible when screening in Steps B.6 and C.6. In general, test *at least* 30 clones. We typically screen 30 clones to obtain one that exhibits both suitably high shRNA induction with the addition of Tc or Dox and low background in the absence of inducer. When we screen stable Tet tTS clones (by the methods described in Section IX.C) at Clontech, we find that a high percentage of clones exhibit >50% knockdown activity (95% and 78% for HeLa and MCF7 cells, respectively).

A. Transfection with ptTS-Neo and Selection (Figure 7)

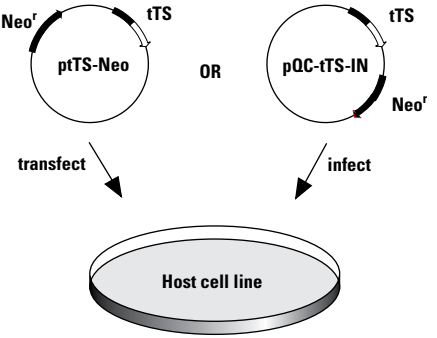
The efficiency of a mammalian transfection procedure is primarily dependent on the host cell line. Therefore, when working with a cell line for the first time, we recommend you compare the efficiencies of several transfection protocols. After choosing a method of transfection, optimize cell density (usually 60–80% confluency), the amount and purity of the DNA, media conditions, and transfection time.

For transfecting HEK 293 cells, we recommend the CalPhos™ Mammalian Transfection Kit (Cat. No. 631312). If a transfection method is already established for your cell line model, proceed with those conditions. It is important to keep optimized parameters constant to obtain reproducible results. For transfecting other cell lines with ptTS-Neo, we recommend using your transfection method of choice with the following protocol.

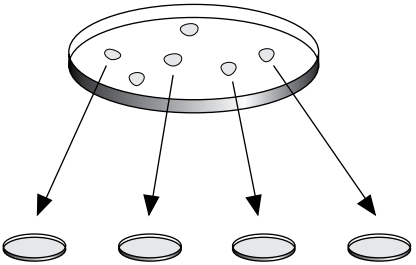
1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method.

IX. Development of Tet tTS Stable Cell Lines *continued*

- Transfect/infect host cell line with regulator plasmid (ptTS-Neo or pQC-tTS-IN)

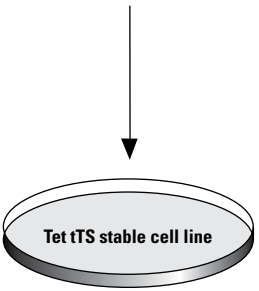


- Select in presence of G418



- Isolate at least 30 G418-resistant clones

- Screen by transient transfection with pSIREN-RetroQ-Tet-Luc and luciferase vector for clones with:
  – Low background (high luciferase activity without Dox or Tc)
  – High shRNA induction (low luciferase activity with Dox or Tc)



- Expand and freeze stocks of Tet tTS stable cell line

Figure 7. Flow chart for developing Tet tTS stable cell lines.

IX. Development of Tet tTS Stable Cell Lines *continued*

2. Transfect the ptTS-Neo Vector by the desired method.

   Note: If desired, the regulator plasmid can be linearized by digestion with a restriction enzyme.

3. Plate transfected cells in ten 10 cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VIII.

4. Allow cells to divide twice (24–48 hr), then add G418 to 400–500 µg/ml.

   Note: The exact concentration of G418 for selection and the optimal plating density may vary from cell type to cell type and with different lots of G418. See Section VIII.A.

5. Replace medium with fresh complete medium plus G418 every four days, or more often if necessary.

   After about five days, cells should start to die. Split the cells if they reach confluency before massive cell death begins.

   After 2–4 weeks, isolated colonies should begin to appear.

6. Isolate large, healthy colonies and transfer them to individual plates or wells. Suspension cultures must be cloned using the limiting dilution technique. When working with adherent cells at Clontech, we generally isolate clones using cloning cylinders or cloning discs.

B. Infection with pQC-tTS-IN and Selection (Figure 7)

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your infection method.

2. Infect cells with pQC-tTS-IN retroviral vector using methods detailed in Section VII.A of the Retroviral GeneTransfer and Expression User Manual (PT3132-1).

3. 24 hr post-infection, plate infected cells in ten 10 cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VIII.A.

4. Allow cells to divide twice (24–48 hr), then add G418 to 400–600 µg/ml.

   Note: The exact concentration of G418 for selection and the optimal plating density may vary from cell type to cell type and with different lots of G418. See Section VIII.A for details.

5. Replace medium with fresh complete medium plus G418 every 3–4 days, or more often if necessary.

   After 4–6 days, cells that have not taken up the plasmid will start to die. Split cells if they reach confluency before massive cell death begins.

6. After 3–4 weeks, isolated colonies should begin to appear. Isolate large, healthy colonies and transfer them to individual plates or wells. Suspension cultures must be cloned by the limiting dilu-

IX. Development of Tet tTS Stable Cell Lines *continued*

tion technique. When working with adherent cells at Clontech, we generally isolate clones using cloning cylinders or cloning discs.

C. Screening Tet tTS Stable Cell Lines

The next step is to perform transient transfection assays with a luciferase expression vector and pSIREN-RetroQ-Tet-Luc to identify G418-resistant clones that meet the criteria for stable tTS cell lines.

1. Pick clones and expand as needed for your particular cell line. Screen clones once they reach 50–80% confluency in a 6-well plate.
2. Trypsinize the cells and split about 1/3 into a single well of a 6-well plate. The cells in this "stock plate" will be propagated depending upon the results of the screening assay.
3. Divide the remaining 2/3 of the cells into 2 wells of a 6-well plate. The following day (ie. once cells have attached) transfect the cells with a 1:2 or 1:1 ratio of luciferase vector:pSIREN-RetroQ-Tet-Luc, using the desired transfection method.
4. Change both plates to fresh media, and incubate one of the wells in the presence of 1µg/ml Dox.
5. After 48–72 hr, assay for knockdown of luciferase activity:

   % knockdown = [with Inducer RLU]/ [without Inducer RLU]
6. Select clones with the highest knockdown (lowest level of inducible RLU) and lowest background (highest level of uninducible RLU) for propagation and further testing.
7. Expand and freeze stocks of each clone as soon as possible after expanding the culture.

X. Development of Double-Stable Cell Lines

A. Screening pSIREN-RetroQ-Tet-shRNA Constructs

It is recommended that you screen for functionality of your oligos prior to developing double stable cell lines with your recombinant pSIREN-RetroQ-Tet-shRNA constructs. shRNA oligos can be screened using either the Knockout RNAi Clone and Confirm RetroQ RNAi Platinum System (Cat. No. 632456) or the Knockout RNAi Clone and Confirm RetroQ RNAi Core System (Cat. No. 632476). These kits provide you with a simple and efficient way to generate candidate shRNA expression cassettes (SECs) for functional screening. SECs are ready-to-transfect PCR fragments generated by one round of PCR with your vector. The yield of SEC from a single PCR reaction is sufficient for at least 10 transfections. For details on these kits please see the Knockout RNAi Clone and Confirm PCR Kits User Manual (PT3779-1).

Alternatively, you can transiently transfect pSIREN-RetroQ-Tet-shRNA DNA (generated in Section VI.C) into a stable tTS cell line (or a premade Clontech tTS Cell Line) and screen for effective knockdown. This screening can be performed after 48 hrs of induction. We recommend that you use the Clone and Confirm System for screening because transfection of pSIREN-RetroQ-Tet vectors creates an environment more prone to promoter interference due to the presence of the LTRs on the plasmid.

If the gene you are silencing does not contain a tag, you will need to design a gene-specific assay to test for its knockdown. Examples of gene-specific assays that can be used include:

- Western blot with an antibody to Protein X
- RT-PCR using Gene X primers. Be sure you can discriminate PCR products generated from genomic DNA from true RT-PCR products.
- Northern blot with Gene X probe
- Functional assay for Protein X
- ProLabel Screening Kits. Our screening kits allow fast and quantititative chemiluminescent measurement of the expression levels of any gene fused to the ProLabel tag. The kits are supplied in two formats, a Creator™ format for genes already cloned into the Creator backbone (Cat. No. 631542) and an In-Fusion™ format for PCR cloning of precise, directional constructs (Cat. No. 631724). For more details, please see the ProLabel Screening Kit User Manual (PT3789-1).

X. Development of Double-Stable Cell Lines *continued*

- Insert shRNA oligo into pSIREN-RetroQ-Tet vector
- Generate recombinant pSIREN-RetroQ-Tet-shRNA virus
- Infect tTS stable cell line with recombinant pSIREN-RetroQ-Tet-shRNA
- Select in presence of hygromycin or puromycin
- Isolate at least 30 hygromycin- or puromycin-resistant clones
- [OPTIONAL] Confirm presence of integrated shRNA in clones by PCR
- Screen by a gene-specific assay for clones with:
  - Low background of shRNA (- Dox/Tc)
  - High induction of shRNA (+ Dox/Tc)

  Possible assays:
  - Western blot using an antibody to Protein X
  - RT-PCR using Gene X primers
  - Northern blot with Gene X probe
  - Functional assay for Protein X
  - Prolabel™ Screening Kits
- Expand and freeze stocks of double-stable inducible RNAi cell lines

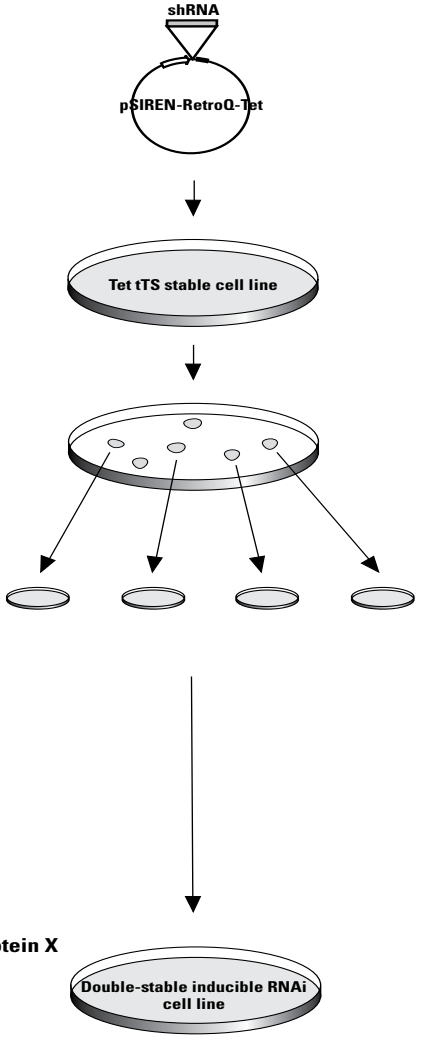


Figure 8. Flow chart for developing double-stable inducible RNAi cell lines.

X. Development of Double-Stable Cell Lines *continued*

Important Note: Knockdown levels are likely to be lower in transient assays than in properly screened stable and double-stable cell lines, since the amount of plasmid in the cell may titrate out the tTS to some degree. Therefore, an apparent lack of induction response in the transient assay should not be the sole reason for aborting your experiments in a particular cell line. In this case titration of the input plasmids may help to give better induction profiles.

B. Infection with pSIREN-RetroQ-Tet Construct and Selection of Double-Stable Cell Lines (Figure 8)

The next step is to *stably* introduce your pSIREN-RetroQ-Tet construct into the stable (or premade) Tet tTS cell line. The goal is to generate a double-stable cell line that gives both low background and high inducible expression of your shRNA. Both expression levels and induction of your shRNA can be profoundly affected by the site of integration. Insertion near an enhancer may result in high basal expression of the shRNA, whereas other insertion sites may result in suboptimal induction. To find the clone with the highest induction and lowest background, we recommend that you grow and analyze as many clones as possible. We typically screen 30 clones to obtain one that exhibits suitably high induction and low background.

1. Grow stable Tet tTS cells to ~80% confluency in complete medium or to a density appropriate for your infection method.
2. Infect with pSIREN-RetroQ-Tet using methods detailed in Section VII.A of the Retroviral Gene Transfer and Expression User Manual (PT3132-1).
3. At 24 hr post-infection, plate infected cells in ten 10 cm culture dishes, each containing 10 ml of the appropriate complete medium, at the optimal density determined in Section VIII.A.
4. Allow cells to divide twice (24–48 hr), then add the appropriate selection agent, hygromycin or puromycin, to the optimal concentration determined in Section VIII.A. For hygromycin the range is generally 200–400 µg/ml and for puromycin it is 0.5–3 µg/ml.

   Note: The exact concentration of antibiotic for selection and the optimal plating density may vary from cell type to cell type and with different lots. See Section VIII.A for details.
5. Replace medium with fresh complete medium containing the selection antibiotic (hyg or pur) every 4 days.

   After about 3–5 days, cells in selection media should start to die. Split cells if they reach confluency before massive cell death begins.

X. Development of Double-Stable Cell Lines *continued*

After 2–4 weeks, hyg-resistant or pur-resistant colonies will begin to appear.

6. Isolate large, healthy colonies and transfer them to individual plates or wells. Isolate as many clones as possible.

C. Screening Double-Stable Inducible RNAi Cell Lines

1. Test isolated resistant clones for Tet-regulated gene silencing by dividing a suitable number of cells in half and testing for Gene X silencing in the absence and presence of Tc or Dox.

   You should generally choose the cell line that gives you the highest overall shRNA expression (and therefore highest suppression of Gene X) in the presence of Dox and lowest background shRNA expression (and highest expression of Gene X) in the absence of Dox.
2. Allow the cells to grow for at least 48 hr, then assay each sample for shRNA expression (via Gene X suppression) using one of the methods described in Section X.A.
3. [Optional] Confirm the presence of integrated pSIREN-RetroQ-Tet-shRNA by performing PCR on chromosomal DNA using primers that will amplify an internal portion of the plasmid.
4. Once you have developed a suitable double-stable inducible RNAi cell line, prepare frozen aliquots to ensure a renewable source of the cells (Section VII.E).

XI. Working with Double-Stable Inducible RNAi Cell Lines

The Tetracyline-controlled system has been established successfully in many cell types, as well as transgenic mice, rats, plants, and yeast. The key in generating successful stable cell lines is to pick and screen carefully reasonable number of clones (we usually pick at least 30 clones at each step). Clonal variation in expression of both the integrated tTS construct and the shRNA construct can be caused by the integration site and by down regulation through, for example, methylation. Such clonal-specific variation is readily mitigated simply by screening more clones.

A. Determination of Effective Concentrations of Dox: The concentrations of Dox listed throughout this protocol are approximate. The optimal concentration may vary with different cell lines and with different antibiotic lots. In general, full activation of shRNA expression with stable cell lines can be obtained with 100 ng–1 µg/ml Dox. Perform a dose-response curve similar to the experiment shown in Figure 6 (Section VIII.B).

B. Loss of Regulation: On occasion, well-characterized double-stable cell lines can lose their responsiveness to Dox. This can occur after changing lots of calf or fetal bovine serum and appears to be due to contamination of some lots of serum with Tc. You can eliminate Tc contamination problems by using the Clontech's Tet System Approved FBS provided with the Inducible RNAi System. This serum has been functionally tested in the Tet Systems to ensure against possible Tc contamination. Additional FBS can be purchased separately (Cat. Nos. 631105, 631101, 631107 & 631106). If you observe a sudden loss of responsiveness, check your serum by performing a dose-response curve as described in Section VII.A of the Tet-Off® and Tet-On® Gene Expression Systems User Manual (PT3001-1). You can also try replating and washing the cells 3 hr later to remove any residual antibiotic that may be interfering with induction control (Rennel & Gerwins, 2002). Loss of regulation can also be due to switching off or methylation of the viral promoter. It is recommended that you subclone and freeze stocks of your cells at various stages to mitigate this risk.

XII. Analysis of Results and Troubleshooting Guide

A. Poor transformation efficiency

Low transformation efficiency can be the result of a problem with the oligonucleotides, ligation, and/or transformation.

Incompatible ends on the insert	Confirm that the ends of the annealed oligonucleotide contain 5' *Bam*H I and 3' *Eco*R I overhangs for proper ligation into pSIREN-RetroQ-Tet vectors.
Ineffective oligo annealing	Verify that the top and bottom strand sequences are correct. To ensure a high amount of dsDNA in the annealing reaction, mix an equal ratio of top and bottom strands. It may be necessary to increase the denaturation temperature (Section VI.A) to increase the yield of annealed oligonucleotide.
Oligos are not full-length	Verify oligonucleotide size using a 12% native polyacrylamide gel. Order PAGE-purified oligonucleotides to ensure a higher percentage of full-length oligonucleotides and increase the chance of cloning a complete and functional insert.
Suboptimal oligo concentration in ligation	Verify the concentration of the annealed oligonucleotide used for ligation. Too little or too much oligonucleotide can affect ligation. To improve ligation efficiency, perform a range of 5- or 10-fold dilutions of the annealed oligonucleotide for use in ligation.
Inactive ligase and/or ligase buffer	Check your ligase and ligase buffer for activity using a different vector and insert. Replace the ligation reagents if they prove inactive.
Suboptimal competent cells	Transform Fusion-Blue Competent Cells using the provided Test Plasmid. Calculate the number of cfu/µg to determine the cells' competency. Handle competent cells gently during transformation and plating. Perform the heat shock step (Step VI.C.4) for precisely 45 sec. Extending this time will drastically reduce cell viability. We have not observed loss or mutation of the annealed oligonucleotides when cloned into pSIREN-RetroQ-Tet vectors and propagated using the recommended conditions. To ensure integrity do

XII. Analysis of Results and Troubleshooting Guide *cont.*

	not overgrow transformed cultures. If planning an overnight culture, inoculate as late as possible in the day using a 1:1,000 dilution of freshly grown stock. Incubate with sufficient shaking to ensure good aeration (250 rpm) and harvest the culture as early as possible the next day to prevent culture overgrowth. Do not serially passage your cultures. In addition to keeping glycerol stocks of transformed cells, we highly recommend keeping DNA stocks of your pSIREN-RetroQ-Tet constructs.
Wrong antibiotic or suboptimal antibiotic concentration	Verify the correct antibiotic and its concentration by checking the Vector Information Packet that accompanies the pSIREN-RetroQ-Tet vector.

B. Poor transfection efficiency

Transfection efficiency can be affected by plasmid purity or transfection conditions. Alternatively, an ineffective pSIREN construct can be misinterpreted as low transfection efficiency.

Poor purity of pSIREN DNA	Ensure the purity of recombinant pSIREN-RetroQ-Tet by isolating all plasmids for transfection using a NucleoBond® Plasmid Midi Kit (Cat. No. 635931) or by CsCl gradient.
Ineffective transfection	The efficiency of a mammalian cell transfection depends primarily on the host cell line. Optimizing the transfection parameters for each cell type is crucial to obtaining consistently successful transfections. Therefore, for each cell type you plan to use, perform preliminary experiments to determine the optimal: 1) amount of transfection reagent; 2) amount and purity of DNA; 3) ratio of transfection reagent to DNA; 4) cell density; 5) transfection incubation time; and 6) media conditions. If you are using Clonfectin Transfection Reagent, see the User Manual for more information.
No detectable gene silencing	You should test a minimum of 3–4 pSIREN-RetroQ-Tet constructs per gene to optimize gene silencing. We provide enough pSIREN-RetroQ-Tet vector in each kit to perform 40 ligations, which allows you to screen for functional shRNA sequences within your gene of interest.

XII. Analysis of Results and Troubleshooting Guide *cont.*

C. Poor knockdown efficiency

Poor knockdown efficiency can be the result of a problem with the host cell line, target sequences, or insufficient suppression.

Unsuitable host cell line	Cell system may not be compatible with the Tet expression system. Perform a transient transfection assay with ptTS-Neo, pSIREN-RetroQ-Tet-Luc, and a luciferase expression vector to ensure functionality in your cell system (Section VIII.B).
Target sequence not optimal	Screen your shRNA oligos by using the Knockout Clone and Confirm RetroQ RNAi Core System or transient transfection of your pSIREN-RetroQ-Tet-shRNA into a stable Tet tTS cell line (Section X.A.)
Insufficient suppression/ leaky background	Tc contamination in media. Use Tet System Approved FBS. Check your serum by performing a dose response curve (Section XI.B).

D. Loss of inducible regulation

Insufficient suppression/ leaky background	Tc contamination in media. See Section C above.
Viral promoter inactivation	Switching off or methylation of the promoter may occur. It is recommended that you subclone and freeze stocks of your cells at all stages.

XIII. References

You can access extensive technical resources and information (including bibliographies) on the RNAi and Tet Systems from their respective product pages at **www.clontech.com.** Clontech also offers extensive online tools to assist you with shRNA oligonucleotide sequence design at:

http://bioinfo2.clontech.com/rnaidesigner/.

Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH). Additional background information on Tet-regulated gene expression systems is available at the site maintained by Dr. Bujard's laboratory:

http://www.zmbh.uni-heidelberg.de/bujard/homepage.html

In addition, IP Merchandisers TET Systems Holding GmbH & Co KG was founded by Dr. Bujard. Up-to-date information on the Tet system technology and licensing issues can be found at the site maintained by TET Systems Holding GmbH & Co KG at:

www.tetsystems.com/

Please note that Clontech is not responsible for the information on, or the maintenance of, these sites.

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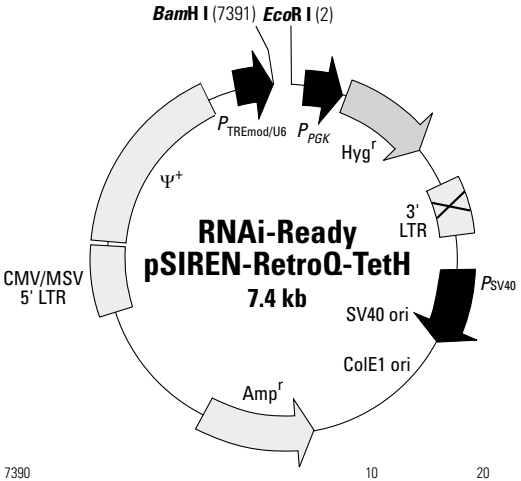
Appendix: Vector Information



7380 7390 10 20 30 40

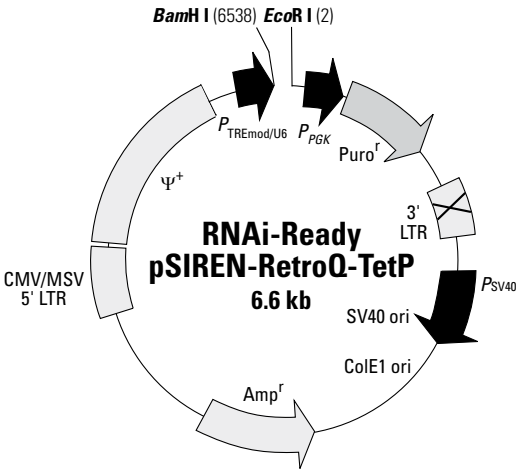
5'-TGTGGAAAGGACGAGGATCC[...shRNA oligo cloning site...]GAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGT-3'

BamH I EcoR I

3'-ACACCTTTCCTGCTCCTAGG[...shRNA oligo cloning site...]CTTAAGATGGCCCATCCCCTCCGCGAAAAGGGTTCCGTCA-5'

Figure 9. Restriction map and cloning site of the RNAi-Ready pSIREN-RetroQ-TetH Retroviral Vector. Unique restriction sites are in bold. RNAi-Ready pSIREN-RetroQ-TetH is a self-inactivating retroviral expression vector designed to express a ds short hairpin RNA (shRNA) under the control of the modified Tet-responsive promoter ($P_{TREmod/U6}$) derived from the P_{TREmod} promoter and the human U6 promoter (P_{U6}). RNAi-Ready pSIREN-RetroQ-TetH is provided as a linearized vector digested with *Bam*H I and *Eco*R I. It is used for targeted and inducible gene silencing when a DNA oligonucleotide encoding an appropriate shRNA is ligated into the vector. shRNA expression is controlled by the tetracycline transcriptional suppressor, ptTS (Freundlieb *et al.* 1999). P_{TREmod} contains a modified Tet response element (TRE_{mod}) which consists of seven direct repeats of a 36 bp sequence that contains the 19-bp tet operator sequence (*tetO*). You can transfect your pSIREN-RetroQ-TetH construct as a plasmid expression vector, or—upon transfection into a packaging cell line—this vector can transiently express, or integrate and stably express a viral genomic transcript containing the $P_{TREmod/U6}$ promoter and the shRNA. The vector contains a hygromycin resistance gene (Hyg^r) under the control of the murine phosphoglycerate kinase (PKG) promoter (P_{PGK}) for the selection of stable transfectants. This retroviral vector is optimized to eliminate promoter interference through self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (Kinsella & Nolan, 1996; Ory *et al.*, 1996; Pear *et al.*, 1996; Yang *et al.*, 1999) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This mechanism may reduce the phenomenon known as promoter interference (Barton & Medzhitov, 2002; Emerman & Temin, 1984) and allow more efficient expression. Viral infection of host cells with recombinant pSIREN-RetroQ-TetH is the preferred delivery method. Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi^+), and tRNA primer binding site. RNAi-Ready pSIREN-RetroQ-TetH also contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria.

Appendix: Vector Information *continued*



7380 7390 10 20 30 40

5'-TGTGGAAAGGACGAGGATCC[...shRNA oligo cloning site...]GAATTCTACCGGGTAGGGGAGGCGCTTTTCCCAAGGCAGT-3'

***Bam*H I** ***Eco*R I**

3'-ACACCTTTCCTGCTCCTAGG[...shRNA oligo cloning site...]CTTAAGATGGCCCATCCCCTCCGCGAAAAGGGTTCCGTCA-5'

Figure 10. Restriction map and cloning site of the RNAi-Ready pSIREN-RetroQ-TetP Retroviral Vector. Unique restriction sites are in bold. RNAi-Ready pSIREN-RetroQ-TetP is a self-inactivating retroviral expression vector designed to express a ds short hairpin RNA (shRNA) under the control of the modified Tet-responsive promoter ($P_{TREmod/U6}$) derived from the P_{TREmod} promoter and the human U6 promoter (P_{U6}). RNAi-Ready pSIREN-RetroQ-TetP is provided as a linearized vector digested with *Bam*H I and *Eco*R I. It is used for targeted and inducible gene silencing when a DNA oligonuceotide encoding an appropriate shRNA is ligated into the vector. shRNA expression is controlled by the tetracycline transcriptional repressor, ptTS (Freundlieb *et al*. 1999). P_{TREmod} contains a modified Tet response element (TRE_{mod}) which consists of seven direct repeats of a 36 bp sequence that contains the 19-bp tet operator sequence (*tetO*). You can transfect your pSIREN-RetroQ-TetP construct as a plasmid expression vector, or—upon transfection into a packaging cell line—this vector can transiently express, or integrate and stably express a viral genomic transcript containing the $P_{TREmod/U6}$ promoter and the shRNA. The vector contains a puromycin resistance gene ($Puro^r$) under the control of the murine phosphoglycerate kinase (PKG) promoter (P_{PGK}) for the selection of stable transfectants. This retroviral vector is optimized to eliminate promoter interference through self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (Kinsella & Nolan, 1996; Ory *et al*., 1996; Pear *et al*., 1996; Yang *et al*., 1999) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This mechanism may reduce the phenomenon known as promoter interference (Barton & Medzhitov, 2002; Emerman & Temin, 1984) and allow more efficient expression. Viral infection of host cells with recombinant pSIREN-RetroQ-TetP is the preferred delivery method. Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi^+), and tRNA primer binding site. RNAi-Ready pSIREN-RetroQ-TetPalso contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria.

Appendix: Vector Information *continued*

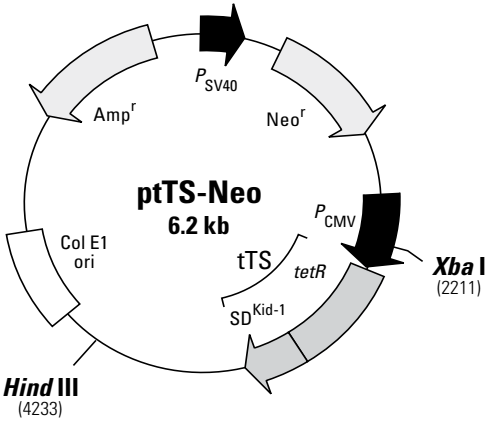


Figure 11. Restriction map of the ptTS-Neo Vector. The ptTS-Neo Vector is an expression vector designed to express the tetracyline-controlled transcriptional suppressor (tTS). The tTS is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}), a powerful transcriptional repressor (Freundlieb *et al.*, 1999; Witzgall *et al.*, 1994). In the *absence* of Dox, tTS binds to the *tetO* sequence in the P_{TREmod} of aTet response plasmid (pSIREN-RetroQ-TetH or pSIREN-RetroQ-TetP) and suppresses expression of the shRNA. As Dox is added to the culture medium, the tTS dissociates from the P_{TREmod}, relieving transcriptional suppression. ptTS-Neo also contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria, as well as a $neomycin^r$ gene for the selection of stable transfectants.

Appendix: Vector Information *continued*

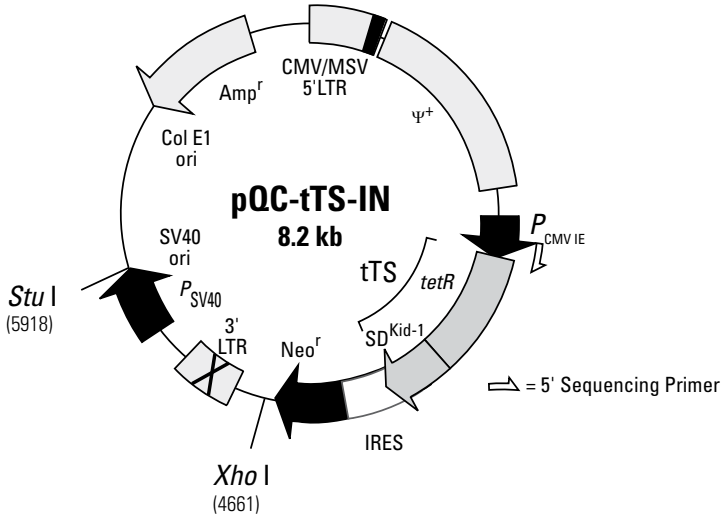


Figure 12. Restriction map of the pQC-tTS-IN Vector. The pQC-tTS-IN Retroviral Vector is a bicistronic expression vector designed to express the tetracyline-controlled transcriptional suppressor (tTS) along with the neomycin selection marker (Julius *et al.*, 2000). Upon transfection into a packaging cell line, this vector can transiently express, or integrate and stably express, a viral genomic transcript containing the CMV immediate early promoter, tTS, IRES and the neomycin resistance gene (Neo^r). tTS and the neomycin resistance gene are cotranslated, via the internal ribosome entry site (IRES), from a bicistronic message in mammalian cells (Adam *et al.*, 1991; Gattas *et al.*, 1991). The tTS is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}), a powerful transcriptional repressor (Freundlieb *et al.*, 1999; Witzgall *et al.*, 1994). In the *absence* of Dox, tTS binds to the *tetO* sequence in the P_{TREmod} of aTet response plasmid (pSIREN-RetroQ-TetH or pSIREN-RetroQ-TetP) and suppresses expression of the shRNA. As Dox is added to the culture medium, the tTS dissociates from the P_{TREmod}, relieving transcriptional suppression. This vector incorporates unique features including: optimization to remove promoter interference and self-inactivation. The hybrid 5' LTR consists of the cytomegalovirus (CMV) type I enhancer and the mouse sarcoma virus (MSV) promoter. This construct drives high levels of transcription in HEK 293-based packaging cell lines due, in part, to the presence of adenoviral E1A (Kinsella & Nolan, 1996; Ory *et al.*, 1996; Pear *et al.*, 1996; Yang *et al.*, 1999) in these cells. The self-inactivating feature of the vector is provided by a deletion in the 3' LTR enhancer region (U3). During reverse transcription of the retroviral RNA, the inactivated 3' LTR is copied and replaces the 5' LTR, resulting in inactivation of the 5' LTR CMV enhancer sequences. This mechanism may reduce the phenomenon known as promoter interference (Barton & Medzhitov, 2002; Emerman & Temin, 1984) and allow more efficient expression. Also included in the viral genomic transcript are the necessary viral RNA processing elements including the LTRs, packaging signal (Psi$^+$), and tRNA primer binding site. pQC-tTS-IN also contains a bacterial origin of replication and *E. coli* Amp^r gene for propagation and selection in bacteria, as well as a neomycin^r gene for the selection of stable transfectants.

Notes

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The CMV promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 assigned to the University of Iowa Research Foundation.

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