A Versatile Viral System for Expression and Depletion of Proteins in Mammalian Cells

RNAi studies

Version 2.2 November 2010

Disclaimer

This system of vectors were derived from the p156RRL-sinPPT-CMV-GFP-PRE/Nhe I vector [1,2]. This vector contains two genetic elements originally missing from the original Invitrogen system: the central polypurine tract (cPPT) from HIV-1, and the Woodchuck hepatitis virus post-transcriptional response element (WPRE). Both elements have been shown to increase expression levels of the transduced gene [3,4,5,6]. I put together this manual by inserting the protocols I currently use to clone, express and produce lentiviruses as well as the genetic maps of the various vectors used for RNAi studies. Another manual dealing with vectors used for expression studies is also available at http://ericcampeau.com/manuals. Please go to http://ericcampeau.com for the latest updates on the vector maps, manuals and protocols. Alternative protocols might work better for different laboratory settings and additional improvements and/or modifications are welcome.

All the vectors described in this manual are available through Addgene (http://www.addgene.org) and the appropriate plasmid numbers are cited through this manual. Additional control vectors were also sent to Addgene, including empty vectors or vectors encoding control shRNAs against the Firefly luciferase or the enhanced green fluorescent protein. These control vectors are described in more details in the appendix.

An article describing the use of these vectors for Expression and Depletion studies was published in PLoS ONE [7]. Please refer to this article or to http://ericcampeau.com for more details.

This system was designed and built at the Lawrence Berkeley National Laboratory (Berkeley, CA) in Dr. Priscilla Cooper's laboratory for the clone numbers without the "w" letter, and at the University of Massachusetts Medical School (Worcester, MA) in Dr. Paul Kaufman's laboratory for the clones starting with the "w" letter. This system was also made possible with helpful discussions and comments from Drs. Christian Beauséjour and Francis Rodier.

Eric Campeau, November 2010

Overview

The viral RNAi system described here can be used to deplete a protein of interest either inducibly or constitutively in various mammalian cells. The vectors can express either small hairpin RNAs (shRNAs) or microRNAs (miRNAs). Please refer to the article published about this system [7] for more details and applications of these vectors. All viruses made with this system are replication-incompetent and can be used to deliver the shRNA or miRNA to mammalian cells for RNA interference (RNAi) analysis. This system uses the Gateway technology (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning.html, for more details about it) and this manual assumes that you are familiar with this technology. ShRNAs or miRNAs are initially cloned into an Entry vector (Figure 1) which can then be recombined via an attL-attR (LR) reaction [8] into a variety of Destination vectors to provide the desired viral backbone and drug selection marker of interest. The recombination reaction is transformed into *Escherichia coli* and there is a dual selection for the desired vector. First, the Entry and Destination vectors carry different drug resistance genes for selection in *E.coli*, kanamycin for the Entry vector, and ampicillin for the Destination vector. The transformation is plated onto ampicillin plates, selecting against any unrecombined Entry vector. Second, the Destination vector contain the ccdB killer gene [9], which is toxic to most *E.coli* strains. The ccdB gene is removed from the Destination vector during the recombination reaction and a susceptible *E.coli* strain is used for the transformation, thereby selecting against the unrecombined Destination vector. In our hands, the efficiency of recombination into the Destination vector is >95% as determined by restriction digestion and analysis of the resulting construct. Because the Entry vectors do not contain any viral-derived repetitive sequences, they can be easily manipulated without significant levels of unwanted deletions which often occur at repetitive sequences propagated in bacteria [10,11,12].

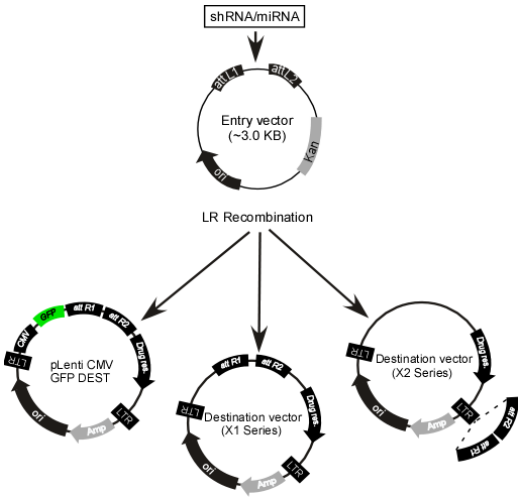


Figure 1. Overview of the RNAi system. The shRNA or miRNA is cloned into the appropriate Entry vector and then recombined with the desired viral Destination vector.

Entry vectors

Six different Entry vectors were generated for RNAi experiments, and are suitable for insertion of either shRNAs or miRNAs (see Appendix for the map of the plasmids):

1. The **pENTR/pSUPER+** plasmid (575-1 and Addgene #17338**)** is based on the pSUPER plasmid [13], which allows the expression of shRNAs under the control of the H1 RNA polymerase III promoter. We inserted a 750 bp stuffer (denoted by the +,

derived from the pTER+ plasmid [14]) between the *Bgl* II and *Hin*d III sites to facilitate cloning of shRNAs downstream of the promoter.

2. The **pENTR/pTER+** plasmid (430-1 and Addgene #17453) is derived from the pTER+ plasmid [14]; this is similar to pSUPER except that a Tetracycline Operator (TO) was inserted at the end of the H1 promoter. The TO allows experimental control of the expression of the shRNA in a "T-REx" cell line (Invitrogen) that expresses the tetracycline repressor (TetR), because the transcription-blocking TetR-TO interaction is relieved by addition of doxycycline or tetracycline [15].

3-5. The **pENTR/pSM2** plasmids are based on the pSM2c plasmid [16,17] for expression of miRNAs, and include the 5' and 3' flanking sequences from the miR-30 miRNA. The original U6 promoter was retained to allow constitutive expression under the control of RNA polymerase III in the **pENTR/pSM2(U6)** version (w51-1, Addgene #17387) of the vector. In the **pENTR/pSM2(CMV)** (w57-1, Addgene #17389) and **pENTR/pSM2(CMV/TO)** (w55-1, Addgene #17388) versions, the U6 promoter was replaced by either a constitutive CMV or a doxycycline-inducible CMV/TO promoter to allow expression under the control of RNA polymerase II. The CMV promoter was reported to generate better depletions in some cases [18] and lower variability than the U6-based promoter when the virus is integrated in the genome [19].

6. Finally, we generated the vector **pENTR/pSM2(CMV-GFP)** (w513-1, Addgene #19170), in which a GFP cDNA was inserted between the promoter and the 5'miR30 sequence, to further enhance the depletion of the target protein [20] and facilitate tracking and sorting of cells expressing the miRNA of interest.

Lentiviral Destination vectors

Using the Gateway technology, each Entry vector can be recombined into multiple retroviral and lentiviral Destination vectors. Every lentiviral Destination vector contains the Woodchuck post-transcriptional regulatory element (WPRE) and the central polypurine tract (cPPT) because these elements increase transduction efficiencies [3,4,5,6]. However, not all combinations of Entry and Destination vectors are meant to be recombined. Because all RNAi Entry vectors contain a promoter, only promoter-less Destination vectors should be used in order to prevent juxtaposition of the two promoters and possibly cause interference. The pLenti X2 series allows insertion of the Entry cassette in their 3'LTR (see below), but this imposes an insert size limit, usually ~ 800bp [21], because larger inserts inactivate the LTR and impair viral integration. Also, some sequences can be detrimental to the function of the 3'LTR [22]. Finally, the pENTR/pSM2 (CMV-GFP) vector encodes GFP and therefore should not be recombined with the pLenti CMV GFP or pLenti X1 GFP-Zeo Destination vectors since they also encode the GFP protein. The compatibilities of each Entry vector for various Destination vectors are summarized in Table 1 as well as represented schematically in Figure 2 for lentiviruses, and Figure 4 for retroviruses. The maps of each Destination vectors are also included in the Appendix.

Table 1: Compatibilities of between the Entry and Destination vectors for RNAi studies.

Entry vector type	Compatible Destination vectors	Incompatible Destination vectors
pENTR/pSM2 Series (non-GFP)	pLenti X1 Series pLenti X2 Series pLenti CMV GFP pQCXI RNAi X2 Series[a]	pLenti PGK Series[b] pLenti CMV Series[b] pLenti CMV/TO Series[b] pQCXI CMV/TO Series[b]
pENTR/pSM2 (CMV-GFP)	pLenti X1 Series	pLenti X2 Series[c] pLenti CMV GFP[d] pQCXI CMV/TO Series[b] pQCXI RNAi X2 Series[c]
pENTR/pTER+ pENTR/pSUPER+	pLenti X1 Series pLenti X2 Series pLenti CMV GFP pQCXI RNAi X2 Series	pLenti PGK Series[b] pLenti CMV Series[b] pLenti CMV/TO Series[b] pQCXI CMV/TO Series[b]

[a]Viral titers will be lower by ~100 fold.
[b]Probable promoter interference.
[c]Insert would be too big for the 3'LTR.
[d]Entry vector contains already a GFP.

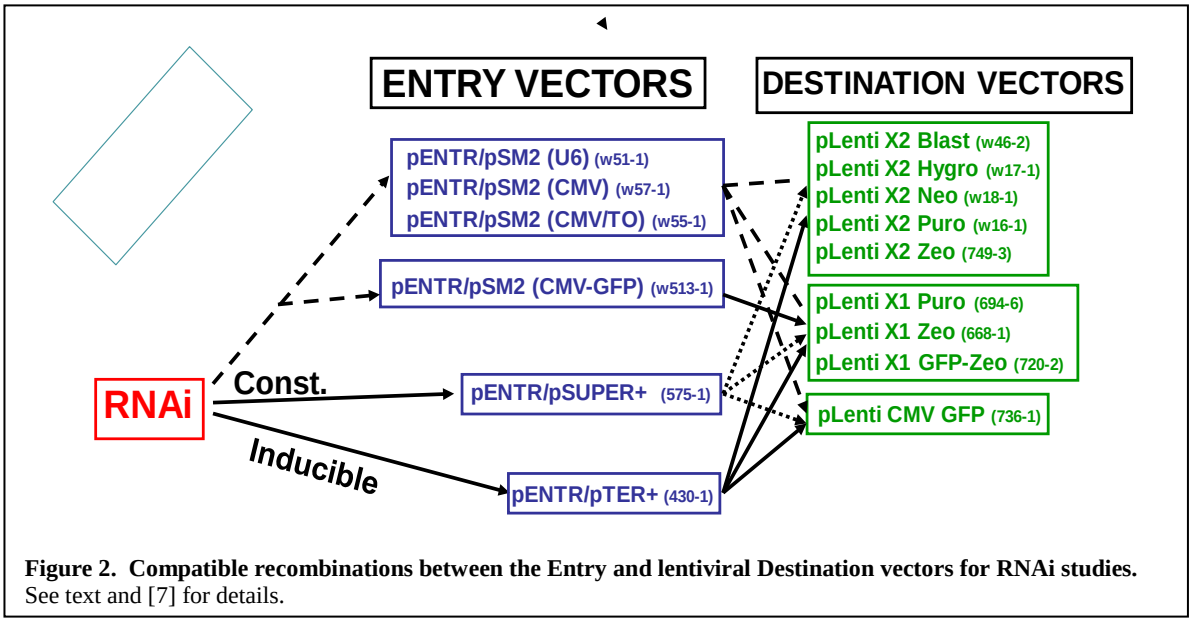


Figure 2. Compatible recombinations between the Entry and lentiviral Destination vectors for RNAi studies. See text and [7] for details.

i. pLenti X1 Destination series

The Gateway Destination cassette was inserted between the cPPT and WPRE elements. One version of the vector confers resistance to puromycin and two others confer resistance to zeocin. Additionally, the pLenti X1 GFP-Zeo vector encodes a GFP-zeocin fusion protein to allow detection of transduced cells by fluorescence. However, the intensity of the GFP fluorescence is lower as a fusion protein which might make it difficult to detect the GFP in live cells or in cell sorting experiments. However, they can readily be detected by immunofluorescence. The article describing these vectors show results obtained using these vectors [7].

ii. pLenti X2 Destination series

Lentiviruses are part of the retrovirus family and have an RNA genome that is reverse-transcribed into DNA before integration into the host genome. During this process, there is a duplication of the 3' LTR to generate the final 5' LTR sequence that is found in the integrated viral genome. Deletions in the U3 region of the 3' LTR (ΔU3) have been used to generate self-inactivating (SIN) vectors for increased biosafety [23,24], and several groups have found that these truncated 3' LTRs tolerate insertion of a small cassette, such as a small promoter (U6, H1, CMV) and an shRNA or miRNA [25,26,27] (Figure 3). Scherr et al. [27] reported that insertion of the H1 promoter cassette in the 3'LTR of the lentiviral backbone resulted in duplication of the H1 RNAi cassette and production of higher levels of shRNAs after viral integration. They also correlated the efficiency of gene knock-down with the levels of shRNA produced by the cell. The pLenti RNAi X2 series has a Gateway destination cassette inserted similarly in the U3 region of the 3'LTR of the lentiviral backbone. After the LR recombination reaction, the ~300 bp cassette can be duplicated over the 5'LTR upon viral integration (Figure 3). The Gateway Destination was inserted in the antisense orientation.

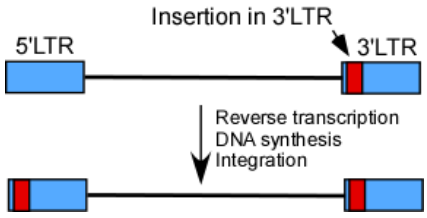


Figure 3. Insertion of the shRNA expression cassette (in red) in the 3'LTR will result in integration of two copies of the cassette per viral integration (pLenti RNAi X2).

Retroviral Destination vectors

In addition to the lentiviral vectors, we also generated retroviral Destination vectors for RNAi studies. Using the same strategy as the pLenti X2 series, we inserted the Gateway Destination cassette into the 3' LTR of the retroviral backbone so we could obtain the expression of two shRNA cassettes upon viral integration into the cellular genome (pQCXI X2 series). Figure 4 and Table 1 summarize the compatibles Entry vectors that can be used with the retroviral Destination vectors. We tested these vectors with the pENTR/pTER and pENTR/pSUPER Series and obtained similar viral titers and depletion efficiencies as the lentiviral backbones [7]. However, we do not recommend these vectors for the pENTR/pSM2(CMV-GFP) and pENTR/pSM2 (CMV) series vectors. In these cases, we obtained lower viral titers [7].

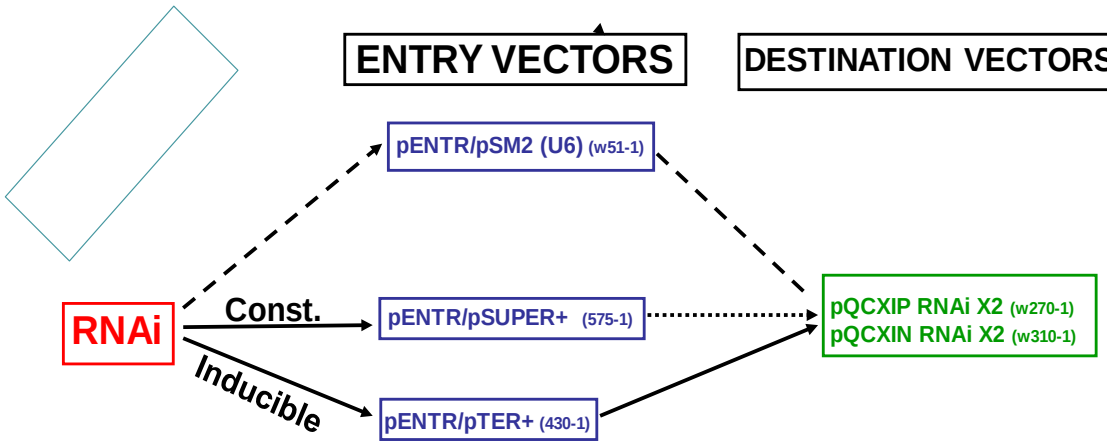


Figure 4. Compatible recombinations between the Entry vectors and the retroviral Destination vectors for RNAi studies. See text and [7] for details.

Advantages of this viral RNAi System:

• Inducible RNAi can be used to deplete a protein essential for the viability of the cell or to prevent secondary changes associated with the loss of protein of interest, as the cell adapt to the loss of the protein.

•The Gateway technology allows the easy transfer of the shRNA/miRNA into the Destination vector, eliminating tedious cloning associated with viral-based vectors.

•Viruses can easily transduce hard to transfect cells for stable long term expression and lentiviruses can also transduce non-dividing cells.

•Pseudotyped virus (e.g. with the VSV-G viral envelope) can transduce a broadened host range.

•As new technologies will emerge, new vectors can be easily added to the system without the need of extensive recloning.

Background

The abilities to express and deplete proteins in mammalian cells are invaluable tools to understand diverse biological processes, both normal and pathological. Traditionally, transfection and electroporation of plasmids into cells have been used to manipulate gene expression. However, not all cell types can be efficiently transfected by these methods, and primary or non-dividing cell types are particularly difficult in this regard [28]. The advent of recombinant retroviruses and lentiviruses [28,29,30,31,32] greatly simplified this task, especially since lentiviruses can easily transduce non-dividing cells. For RNA interference (RNAi), plasmid-based systems used to express shRNAs or miRNAs allowed long term depletion of the protein of interest, compared to the transfection of small interfering RNAs (siRNAs) which results in only transient depletion. Several laboratories and companies have therefore developed viral vectors for the delivery of cDNAs and/or shRNAs or miRNAs to a wide variety of cells [8,17,20,33,34,35,36,37,38,39,40,41]. Even though these vectors have proven extremely useful, there are limited drug selection markers available, and different vector backbones can be required to express cDNAs or shRNAs/miRNAs. We started developing vectors on a "as needed" basis depending on the types of experiments we were doing. At the time of publication of the article describing this system [7], we had 59 different vectors for expression and depletion of proteins either constitutively or inducibly, in mammalian cells. We sought to extend the versatility of viral vectors by developing third generation (Tat-independent), self-inactivating (SIN) lentiviral vectors with an expanded range of drug selection markers, promoters, and epitope tags. Furthermore, we wanted to improve their flexibility, allowing the investigator to rapidly change promoters and/or drug selection markers without extensive recloning. These vectors are based on the Gateway System [8] (Invitrogen) whereby a cDNA, shRNA or miRNA is cloned into an Entry vector which is recombined *in vitro* with the viral Destination vector of choice. This system offers two main advantages: First, the recombination is very efficient and rapid (1-3 h), eliminating most of the stability problems that often arise during traditional cloning in a bacterial host [10,11]. Second, the extensive Destination vector library can be further modified to accommodate future technologies without the need for recloning cDNAs, shRNAs or miRNAs.

Generation of shRNAs. Small RNAs such as tRNAs are transcribed by the RNA polymerase III (pol III). Pol III transcripts are terminated by a stretch of 5-6 thymidines [42] and do not encode any poly A tails. Therefore, pol III promoters such as the H1 or U6 promoters are ideal to generate shRNAs. To generate a shRNAs against the gene of interest, an oligonucleotide linker is subcloned downstream of the H1 promoter in the pSUPER or pTER Entry vectors. To obtain a potent shRNA, the following features are part of the linker: a 19-29 nucleotides sequence derived from the target mRNA followed by a miRNA-derived loop (which was shown to generate better depletion efficiencies [43,44]), the complement of the 19-29 nucleotide sequence and the pol III termination signal (TTTTT). Upon transcription, the RNA molecule folds back on itself to form a hairpin that is then recognized and processed by the Dicer enzyme into a 21-23 nucleotide siRNA duplex, and incorporated into the RNAi-silencing complex (RISC).

Generation of miRNAs. Although miRNAs can also be transcribed by pol III promoters [45], they are normally transcribed by the RNA polymerase II (pol II) in the cell [46,47]. The pENTR/pSM2 series of vectors used to generate miRNAs are based on the pSM2c plasmid [16,20]. These plasmids include the 5' and 3' flanking sequences from the miR-30 miRNA and can be transcribed by either pol II (from the CMV or CMV/TO promoters) or pol III (from the U6 promoter). In this system, an oligonucleotide encoding the target against the mRNA of interest is PCR-amplified using two linker primers and subcloned into the Entry vector. Upon transcription, the RNA molecule will adopt the secondary structure of the miR-30 miRNA and then be processed by the enzyme Drosha through the normal pre-miRNA to miRNA pathway, exported out of the nucleus by exportin-5, and further processed into the RISC.

Mechanism for inducible expression of shRNAs/miRNAs (T-REx system). The shRNA/miRNA can be expressed inducibly, which can be useful if the protein of interest is essential for the viability of the cell. Also, constitutive depletion of a protein might elicit a response from the cell to compensate by activating/de-activating alternate pathways in order to minimize the consequences of the loss of the protein. Therefore, an initial strong response to the loss of a protein might be compromised or severely reduced due to the adaptation of the cell. This viral system uses the T-REx system from Invitrogen to induce either shRNAs or miRNAs based on the tetracycline resistance operon [48,49,50]. As shown in Figure 5, the promoter (H1/TO in the case of shRNAs and CMV/TO in the case of miRNAs) contains the tetracycline operator (TetO or TO) sequence which can be bound by the tetracycline repressor (TetR) to repress transcription. TetR can only bind to the TetO sequence in the absence of its ligand, either tetracycline or its derivative, doxycycline. Doxycycline, is more stable than tetracycline and can induce gene expression at lower concentrations. Therefore, we opted for the use of doxycycline to induce RNAi. Addition of doxycycline to the culture media induces a conformational change in TetR upon binding of doxycycline and prevents the repressor from binding to the operator, therefore inducing transcription of

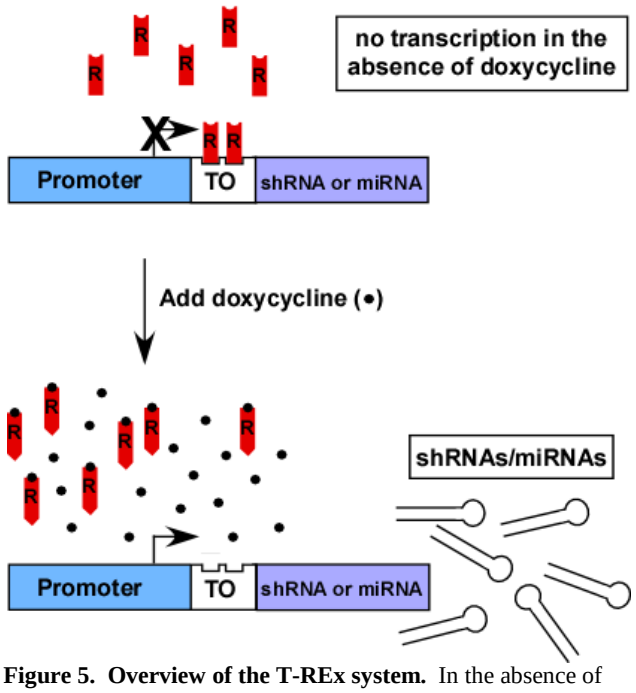


Figure 5. Overview of the T-REx system. In the absence of doxycycline, the tetracycline repressor (R) binds to the tetracycline operator (TO) and prevents transcription from the promoter. Upon addition of doxycycline, it binds to the repressor and alters its conformation so it cannot bind to the operator, hereby inducing transcription of the shRNA/miRNA.

the shRNA/miRNA. Several T-REx cell lines are available through Invitrogen (see Invitrogen's web site for more details). As discussed below, T-REx cell lines can easily be generated using the pLenti CMV TetR Blast (716-1, Addgene #17492)

The pLenti CMV TetR Blast (716-1, Addgene 17492) lentiviral vector can be used to create T-REx cell lines. Sometimes, the cell line of choice is not available in the T-REx version. A relatively easy way to generate such a cell line is to transduce it with the pLenti CMV TetR Blast lentivirus. We used this virus to generate several T-REx cell lines in either primary fibroblasts or transformed cells [7]. On some occasions, it can be useful to screen colonies in order to obtain a clone that ensures either minimal or no leakiness of the shRNA/miRNA. However, in some cases where the cell line as a limited lifespan, such colony isolation cannot be done and one has to work with the entire population. In order to generate a good T-REx cell line, it is critical to have a good TetR to TO ratio, i.e. that the repressor is saturating compared to the operator. If there are not enough repressor molecules, not all promoters will be occupied and leaky transcription will occur. Therefore, a relatively low multiplicity of infection (MOI) has to be done for the transfer vector, that is, the vector containing the shRNA/miRNA. See the appendix for a map of the vector.

Not any vectors can be used to generate an inducible RNAi cell lines. In order to generate an inducible cell line, the TetR must be expressed and the promoter driving expression of the shRNA/miRNA requires a TO. In table 2, the capacity of each vector to generate inducible expression of an shRNA/miRNA in a T-REx cell line is shown. In the absence of TetR, all vectors will show constitutive expression of the shRNA/miRNA.

Table 2: Capacity of RNAi vectors to generate an inducible shRNA/miRNA in a T-REx cell line.

Vector	Inducible expression in a T-REx cell line
pENTR/pSUPER+	NO
pENTR/pTER +	YES
pENTR/pSM2(U6)	NO
pENTR/pSM2 (CMV)	NO
pENTR/pSM2 (CMV/TO)	YES

The RNAi pathway, heterochromatin formation, and proper controls. Traditionally, the role of the RNAi machinery in the cell has been perceived as a possible mechanism to prevent genomic instability from viruses and transposable elements. However, recent reports suggest a much wider role of RNAi in heterochromatin assembly in yeast, flies and vertebrates [51,52,53]. Loss of Dicer in chicken DT40 cells results in defective centromeric heterochromatin formation, causing premature chromosome separation in mitosis. Dicer-deficient mouse embryonic stem cells were viable but showed various defects in differentiation and epigenetic heterochromatin silencing [54]. Centromeric RNA transcripts are processed by the RNAi machinery into the RISC complex. The RISC complex then recruits the Suv39h histone methyltransferase to the centromere where it methylates histone H3 at lysine 9 (H3-K9) thereby enabling interaction of the

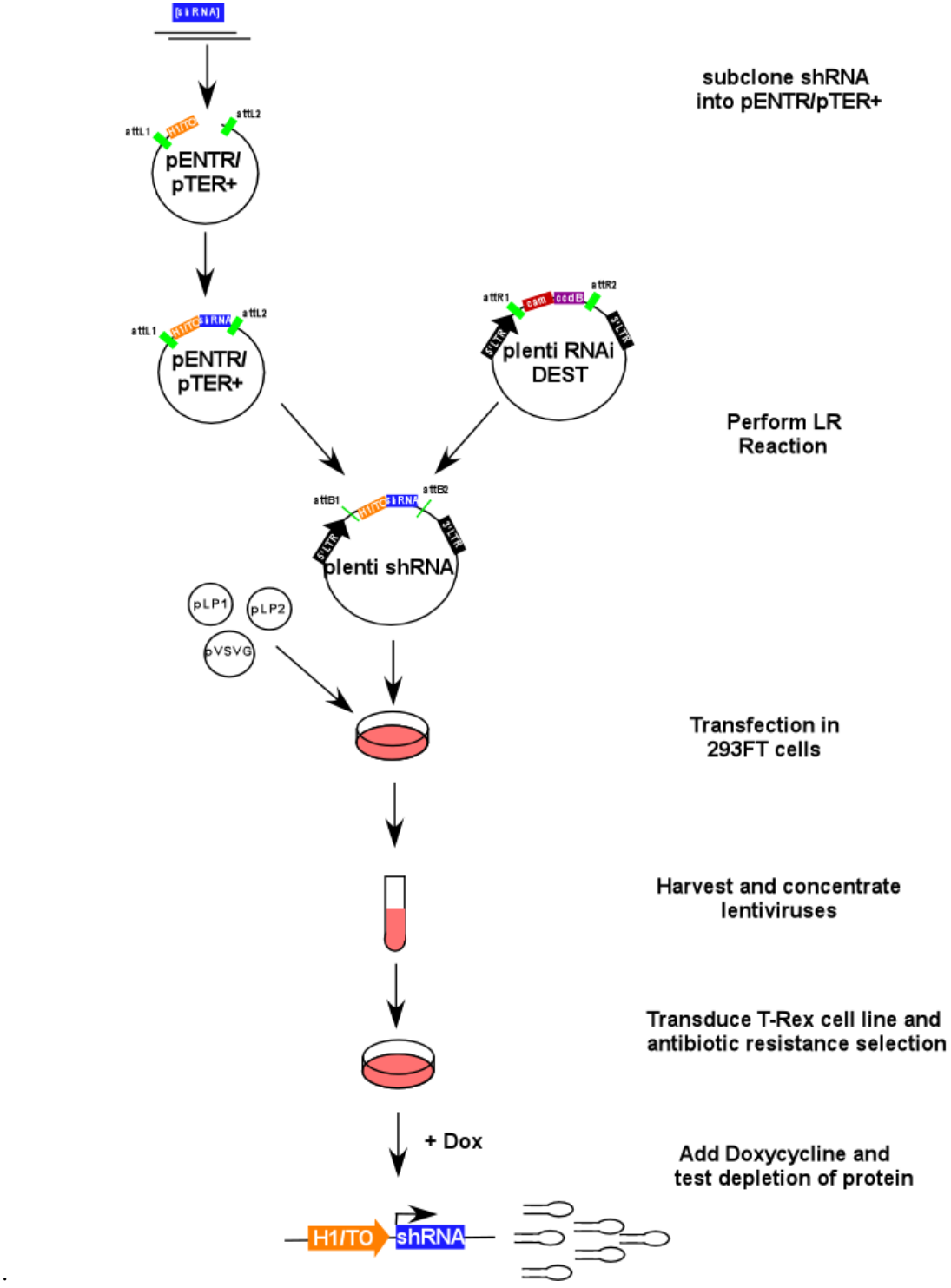
histone with the heterochromatin protein 1 (HP1)-Cohesin complex. Depletion of Dicer disrupts the centromeric localization of HP1 into a diffuse distribution. This proposed mechanism of RNAi still needs further testing and identification of other interacting partners or pathways. Also, mutations in DICER1, the ribonuclease involved in processing shRNAs and miRNAs, have recently been implicated in familial pleuropulmonary blastoma, a rare pediatric lung tumor [55]. These examples show that the RNAi machinery is involved in various cellular processes, and that expression of shRNAs or miRNAs can disrupt these processes through saturation of the RNAi machinery. Evidences of such saturation have been reported [56,57], and experiments have to be carefully designed to monitor for such an effect. We designed control vectors to deplete two control proteins that are often used in various experimental settings: the enhanced green fluorescent protein (GFP) and the firefly luciferase (LUC). These vectors were also sent to Addgene and their maps are in the appendix section. Every RNAi experiment should use an shRNA against either GFP or LUC in the same backbone as the experimental shRNA as negative control. Also, two independent shRNAs/miRNAs against the candidate mRNA should be tested to rule out off-target effects. We do not recommend depletion of a protein normally present in the cell as it could have unforeseen effects on the cell. Some companies recommend the depletion of lamin A as a negative control; however, defects in lamin A has been associated with the Hutchinson-Gilford progeria syndrome where cell lines derived from patients showed abnormal nuclear membrane, loss of peripheral heterochromatin and clustering of nuclear pores [58,59].

Our vectors do not encode the HIV Tat protein. With each new generation of lentiviral packaging vectors, fewer HIV proteins are co-expressed with the recombinant viral genome, resulting in greater biological safety. In second generation vectors, most of the HIV proteins not required for viral replication are removed, with the exception of the HIV Tat protein which is required for transcription of the viral genome from the 5' LTR. It is provided by the packaging plasmid [60,61,62] and the Tat protein is produced and secreted into the media by the packaging cell line. Transfection of a Tat-expressing plasmid results in the secretion of biologically active Tat [63,64]. In third generation vectors, the 5' HIV LTR was replaced by either a hybrid 5' CMV/LTR or 5' RSV/LTR to allow transcription of the viral genome in the absence of the HIV Tat protein without a decrease in viral titers [24]. Accordingly, third generation packaging plasmids do not express the HIV Tat protein. Because several studies have pointed out the toxicity of the HIV Tat protein and that it interferes with several cellular processes [65,66,67,68,69,70,71,72,73,74], we used third generation vectors as the basis for our constructs and all of our lentiviral vectors have the hybrid 5' RSV/HIV LTR and do not require the HIV Tat for transcription of the viral genome. We still recommend transduction with vectors encoding a control shRNA/cDNA or an empty vector at similar titers to monitor possible toxic effects of the transduction.

Our vectors should be handled as a biosafety level 2 environment. Despite the inclusion of safety features such as self-inactivation (SIN) or the removal of most of the HIV proteins, the lentiviruses produced with this system can still pose some biohazardous risk since they can transduce primary human cells. For this reason, **we highly**

recommend that you treat lentiviral stocks generated using this system as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination. Furthermore, exercise extra caution when creating lentivirus that express shRNA targeting human genes involved in controlling cell division (*e.g.* tumor suppressor genes). For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded from the Web at the following address: http://www.cdc.gov/biosafety/publications/bmbl5/

Experimental Outline



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Methods

I- Cloning of shRNAs

1- Selection of a target sequence and synthesis of the oligonucleotides.

Analysis of the candidate cDNA for polymorphisms and alternative splice sites. In order to make sure the RNA hairpin will recognize its target sequence, the various splice variants and polymorphisms of the gene of interest have to be catalogued. I usually do a survey of the published sequences on ENTREZ and dbEST databases.

Generation of candidate sequences. The first step I normally use in deciding which sequence could generate a good shRNA is a general survey of the literature. I go through Medline, google, google scholar, and look if another group has generated a shRNA against my gene of interest. I select papers that measure depletion efficiencies by either RT-PCR or Western blot, and also I make sure they are giving the target sequence they used for their RNAi experiments. If no articles give a satisfactory shRNA sequence, I usually use the Dharmacon siRNA design center or the Invitrogen algorithm. I paste the sequence of the cDNA and go through the steps to get candidate sequences. I eliminate sequences that fall in regions of polymorphisms or splice variants (see above). Then I choose sequence that seems likely to work. Other company that have design softwares: Ambion, Sequitur, Invivogen, Imgenex, GenScript, Mirus Biotech, Promega and Qiagen.

Sequence of the hairpin oligos and synthesis options. I use the following template:

Sense oligo: GATCCCXXXXXXXXXXXXXXXXXXXXGTGTGCTGTCCXXXXXXXXXXXXXXXXXXXXTTTTTGGAAA
Antisense: AGCTTTTCCAAAAAXXXXXXXXXXXXXXXXXXXXGGACAGCACACXXXXXXXXXXXXXXXXXXXXGG

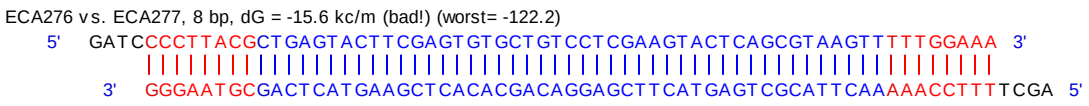
The "X" represents the target sequence for your gene. The GTGTGCTGTCC is the sequence of the miRNA loop which has been reported to generate better depletion efficiencies [43]. The five "T"s (underlined) are the transcription termination signal for the RNA polymerase III. I usually verify the sequences with the program "PrimerSelect" (DnaStar) to make sure that the sense primer can indeed form an hairpin and that the two oligonucleotides are complementary and no mistakes have been made in the oligo. The length of the oligo should be 65 bp. For example, the oligonucleotides that were designed against the Firefly luciferase are shown below:

SENSE: ECA276
5'-GATCCC**CTTACGCTGAGTACTTCGA**GTGTGCTGTCC**tcgaagtactcagcgtaag**TTTTTGGAAA-3'

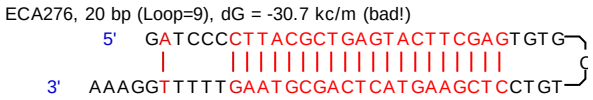
ANTISENSE: ECA277

5'-AGCTTTTCCAAAAA**CTTACGCTGAGTACTTCGA**GGACAGCACAC**tcgaagtactcagcgtaag**GG-3'

Using the PrimerSelect program, the two primers form a perfect primer-pair dimer and the overhangs are compatible with the overhangs of the Bgl II and Hind III digested DNA respectively:



Oligonucleotide ECA276 also forms a hairpin:



I order these oligos from Invitrogen, **at a scale of 25 nmole, desalted (<u>not</u> HPLC purified) and unphosphorylated at their 5' end.**

NOTE: To make the cloning easier, the oligos can be ordered phosphorylated and the pENTR/pTER+ plasmid can be treated with calf intestine phosphatase (CIP) to reduce further the background from the empty vector. In my hands, I now skip that step since it saves on the costs for the oligonucleotide synthesis.

2-Generating an Entry Clone in pENTR/pTER+ or pENTR/pSUPER+

Propagating the entry vector

The pENTR/pTER+ (430-1, Addgene #17453) is derived from the pTER+ plasmid [14] whereas the pENTR/pSUPER+ (575-1, Addgene #17338) plasmid is derived from the pSUPER plasmid [13]. These plasmids can be propagated in a variety of *E.coli* strains under kanamycin selection. I normally use the TOP10F' strain, which is available from Invitrogen.

Insertion of the oligonucleotides in the pENTR/pTER+ plasmid. NOTE: Both plasmids contain a stuffer of 757 bp (denoted by a + sign) between the Bgl II and Hind III sites to facilitate cloning.

1. Digest pENTR/TER+ or pENTR/pSUPER+ plasmid with Bgl II/Hind III (in 1X NEB #2, usually 10 µl of miniprep DNA (~ 1 µg) with 10 units of each enzyme in a final volume of 20 µl). Digest for at least 90 minutes. *If the oligonucleotides are phosphorylated, add 2 µl of calf intestine phosphatase (CIP) after the digestion, and incubate at 37°C for 15 minutes.* After digestion, run a 1% agarose gel to cut out the digested plasmid. The running time will depend on the type of gel apparatus used and the size of the gel. I normally use a 15 cm long gel (100 ml of agarose) and it takes ~90 minutes.

2. While the gel is running, make a 1:500 dilution of the oligo stocks in water (stock is originally ~ 1 µg/µl).

3. Mix the diluted oligos together, heat at 94°C for 5 minutes in the PCR machine and slow cool to room temperature (usually done by turning off the machine and leaving the samples to reach room temperature).

4. Let the gel migrate to obtain a good separation between the 2kb and 3kb band of the molecular weight marker. After the migration, the gel should look as follows:

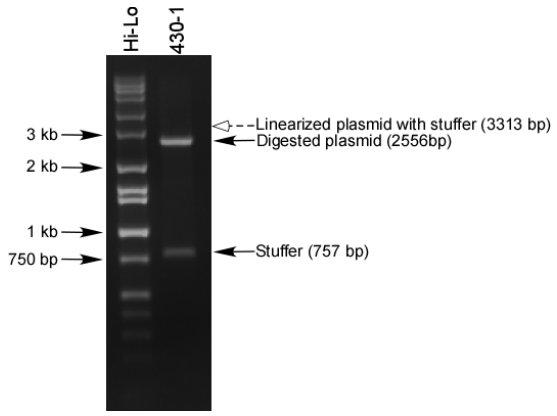


Figure 7. Digestion of pENTR/pTER+ with Bgl II/Hind III. Upon digestion, two bands (2556 bp and 757 bp) are expected. If the digestion is incomplete, an additional 3313 bp will be seen. Cut the 2556 bp band and proceed to geneclean. Hi-Lo DNA ladder is from Bionexus.

Cut the band corresponding to the linearized plasmids (without the stuffer), geneclean [Q-Biogene] it according to standard procedures (use 5 μl of glassmilk) and elute in 20 μl of elution buffer. (A geneclean protocol is included in Appendix).

5. Mix plasmid and oligos as follows: 10 μl of genecleaned, linearized plasmid
8 μl of oligo mixture
2 μ of 10X T4 DNA ligase buffer
1 μl of T4 DNA ligase

6. Incubate overnight at 15^{o}C and transform in TOP10F' using standard protocols (a transformation protocol is included in the appendix).

Verification of the clones by restriction digest and sequencing. Pick colonies and let them grow overnight under kanamycin selection, isolate the plasmid using a miniprep kit, and digest the plasmids with Eco RI (Eco RI cuts before and after the H1/TO cassette, generating a 325bp band when the oligonucleotides are inserted). The original pTER plasmid can be used as a negative control since it does not contain any insert. Also, the size of the cassette with the insert (325 bp) can be easily distinguished from the empty cassette (260 bp) using the appropriate molecular weight standard on a 2% agarose gel or a 5% polyacrylamide gel. For the agarose gel, it should look similar to Figure 8:

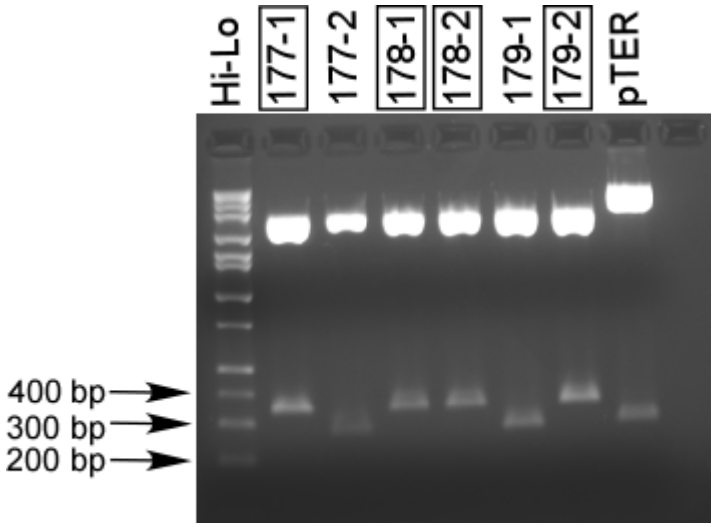


Figure 8. 2% agarose electrophoresis of the candidate clones digested with Eco RI. Insertion of the shRNA oligonucleotides will result in a band migrating around 325bp as opposed to 260 bp for the empty vector. Here, pTER is used as a negative control since it does not contain any shRNA. Clones 177-1, 178-1, 178-2 and 179-2 are good whereas clones 177-2 and 179-1 are empty. Clone 177-1 was sent for sequencing.

Always send the Entry vector with the shRNA for sequencing to make sure no mistakes were made during the oligonucleotides synthesis. Use the ENTRY forward and/or reverse sequencing primers. Some sequencing companies have special sequencing protocols for shRNAs. After sequencing, a typical chromatogram will look as follows (Figure 9):

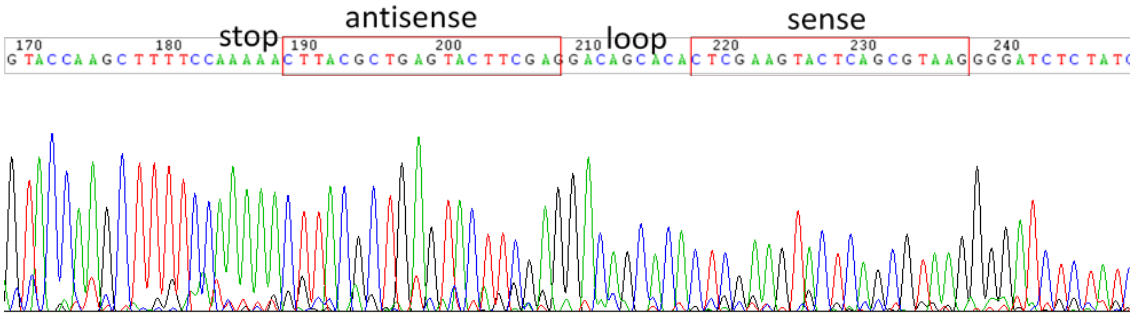


Figure 9. Sequencing of clone 177-1 with the ENTRrev primer. The position of the sense and antisense recognition sequence for the shRNA against the firefly luciferase is shown as well as the miRNA loop and termination signal for RNA polymerase III.

In this case, the Entry reverse primer was used for the sequencing reaction and therefore the antisense strand was sequenced. The plasmid 177-1 is good and was sent to Addgene (Addgene.org) as pENTR/pTER shLUC (w177-1) which is plasmid #17472 in their database. Two other control shRNA plasmids were also sent to Addgene: pENTR/pTER shEGFP #1 (628-2, plasmid #17470) and pENTR/pTER shEGFP #2 (728-2, plasmid #17471) encoding shRNAs against the enhanced GFP protein.

Note for sequencing shRNAs: As shown in Figure 7, there was a slight decrease in the signal intensity as the sequencing polymerase was going through the hairpin sequence (compare the height of the peaks before and after the hairpin). In this case, the loss of signal intensity was minimal and the corresponding shRNA sequence could easily be read. In some cases, the hairpin is a strong block to the sequencing polymerase and there is a sudden drop in signal intensity. Sequencing both sides of the hairpin using the forward and reverse primers can solve the problem in some cases and allow reading of the entire sequence. In some cases, special sequencing protocols for hairpin sequences can also solve the problem. Different sequencing companies use different protocols and in our experience, some companies are clearly better than others.

Once the Entry clone is ready, please proceed to step 3: Recombination into a suitable Destination vector (page 25).

II- Cloning of miRNAs

1- Selection of a target sequence and synthesis of the oligonucleotide.

The pENTR/pSM2 Series of vectors are based on the pSM2c vector from Open Biosystems (http://www.openbiosystems.com)[16,20]. The strategy used to clone miRNAs is different than the one used for shRNAs in the previous section. First, an oligonucleotide of 97bp is synthesized with a Xho I linker sequence at its 5' end and an Eco RI linker sequence at its 3'end. The 97 bp oligo will be amplified by PCR using the Xho I and Eco RI linker primers (Figure 10).

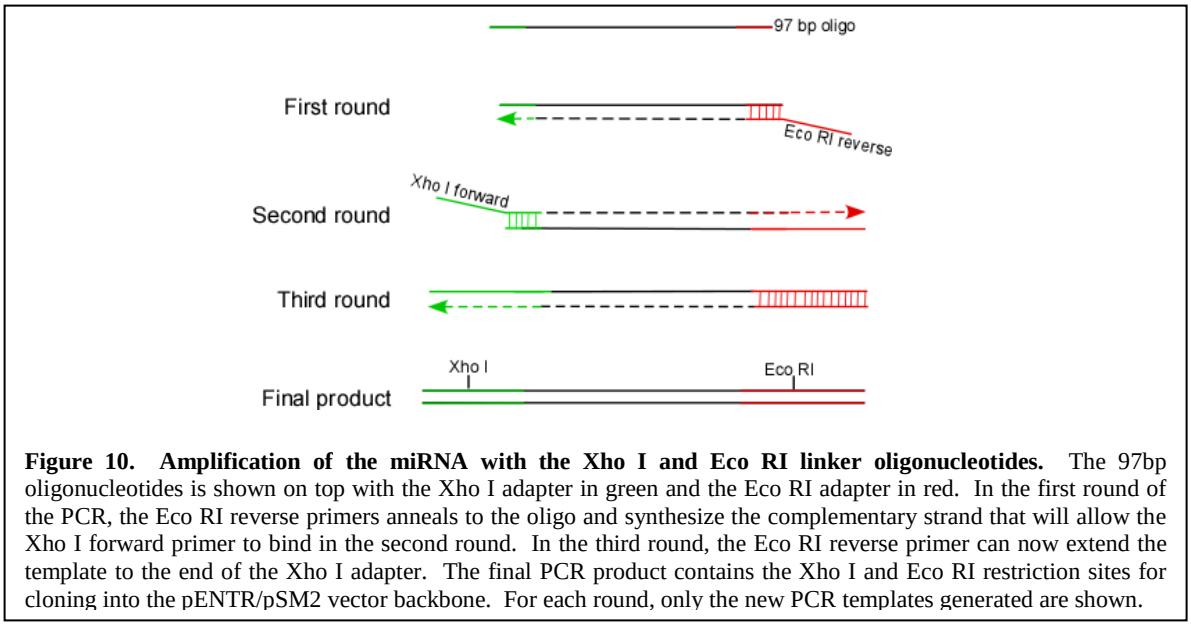


Figure 10. Amplification of the miRNA with the Xho I and Eco RI linker oligonucleotides. The 97bp oligonucleotides is shown on top with the Xho I adapter in green and the Eco RI adapter in red. In the first round of the PCR, the Eco RI reverse primers anneals to the oligo and synthesize the complementary strand that will allow the Xho I forward primer to bind in the second round. In the third round, the Eco RI reverse primer can now extend the template to the end of the Xho I adapter. The final PCR product contains the Xho I and Eco RI restriction sites for cloning into the pENTR/pSM2 vector backbone. For each round, only the new PCR templates generated are shown.

The sequence of the primers for the PCR is as follows:

5'-miR30PCRxhoI-F (forward primer): 5'-CAGAAGGCTCGAGAAGGTATATGCTGTTGACAGTGAGCG-3'
3'-miR30PCREcoRIF (reverse primer):5'-CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA-3'

To design the 97bp oligonucleotides, I normally use the RNAi central website at: http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA. There, you can enter the sequence or accession number of your gene and retrieve the oligo sequence. For example, to design an miRNA against Asf1a (accession # NM_014034), the following oligonucleotide sequences were obtained:

```
22_mer: CTCACTAAATGTCATGTTAGAA
NM_014034.2 Start position: 909
TGCTGTTGACAGTGAGCGATCACTAAATGTCATGTTAGAATAGTGAAGCCACAGATGTATTCTAACATGAC
ATTTAGTGAGTGCCTACTGCCTCGGA

22_mer: TCCACATTAATTGGGAAGATAA
NM_014034.2 Start position: 782
TGCTGTTGACAGTGAGCGCCCACATTAATTGGGAAGATAATAGTGAAGCCACAGATGTATTATCTTCCCAA
TTAATGTGGATGCCTACTGCCTCGGA
```

22_mer: AACTCACTAAATGTCATGTTAG
NM_014034.2 Start position: 907
TGCTGTTGACAGTGAGCGCACTCACTAAATGTCATGTTAGTAGTGAAGCCACAGATGTACTAACATGACAT
TTAGTGAGTTTGCCTACTGCCTCGGA

Alternatively, I search the Open Biosystems website for shRNAmir against Asf1a and I can obtain more sequences such as this one (catalog #RHS1764-9098480):

TGCTGTTGACAGTGAGCGAGGTCACAAGATTCCACATTAATAGTGAAGCCAC
AGATGTATTAATGTGGAATCTTGTGACCCTGCCTACTGCCTCGGA

In every case, the sequence of the end of the Xho I forward primer is the same as the beginning of the 97bp oligo, and the end of the Eco RI reverse primer is complementary to the end of the 97bp oligo. If your University/Institute has access to the shRNA/shRNAmir libraries from Open Biosystems, it can be advantageous to order the clones from them to test if the miRNA can deplete your protein of interest. The shRNAmir cassette can easily be subcloned into the pENTR/pSM2 vector as outlined below.

2-Generating an Entry Clone in the pENTR/pSM2 Series

PCR amplification. Amplify the oligonucleotides with the 5' Xho I forward and 3' Eco RI reverse miR primers. I normally use the following PCR conditions: 25 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 minute. During the PCR, the Xho I and Eco RI adapters are added to the oligonucleotides to allow cloning into the pENTR/pSM2 backbone as shown schematically in Figure 10.

1. Run a 2% agarose gel and cut the PCR product which should be 137 bp. A typical result is shown in Figure 11.

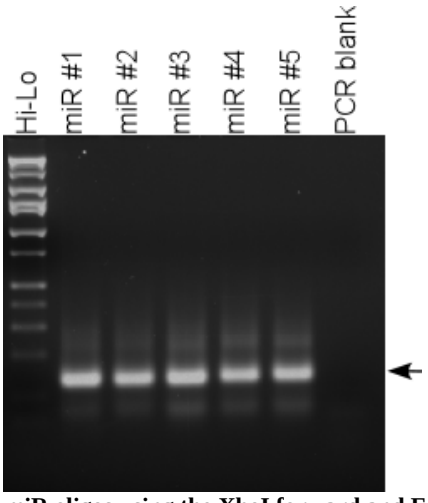


Figure 11. PCR of miR oligos using the XhoI forward and Eco RI reverse linker primers. The PCR band was cut, genecleaned and subcloned into the pCR2.1 plasmid (TA cloning kit, Invitrogen).

2. Purify the band by geneclean and elute the DNA in 10 ul of water. A geneclean protocol is included in the appendix. Use 6 µl of the purified PCR product for the TA cloning ligation (final ligation volume of 10 µl). The protocol I use for TA cloning is included in the appendix. For more details about TA cloning and other TA cloning-derived products, please visit Invitrogen's web site at http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/PCR-cloning.html . Ligate overnight at 15^{o}C.

3. Transform 4 µl of the ligation into *E.coli* TOP10F' using standard procedures and plate on either X-GAL/Ampicillin or X-GAL/Kanamycin plates. A transformation protocol is included in the appendix.

4. Inoculate white colonies, perform minipreps and digest with Eco RI/Xho I to confirm the presence of the insert. Again, run the digested plasmids on a 2% agarose gel. Now, a band migrating at 108 bp is seen positive clones, as shown in Figure 12. The band is smaller because parts of the PCR adapters are retained in the pCR2.1 backbone. The positive clone can be sent for sequencing at this step or once the miRNA is subcloned in the Entry vector.

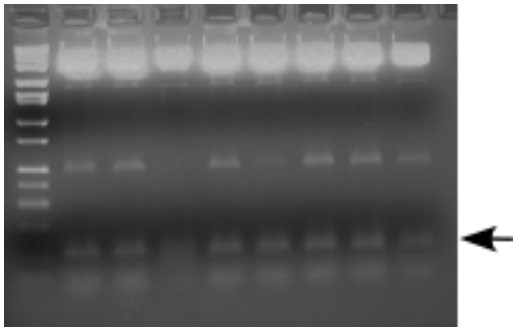


Figure 12. Xho I/Eco RI digestion of the miRNA from the TA pCR2.1 cloning vector. The 108 bp band to cut is indicated by the arrow. The other bands are coming from the pCR2.1 backbone.

5. Cut the 108 b band and ligate it with the pENTR/pSM2 vector digested with Xho I/Eco RI. Transform *E.coli* TOP10F' and analyze minipreps by digesting with Xho I/Xba I and running a 2% agarose gel. The empty pENTR/pSM2 backbone will have a band of 154 bp where as the vector containing the insert will have a band of 262 bp as shown in Figure 13.

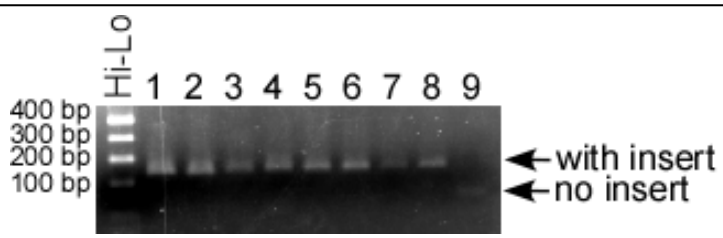


Figure 13. Xho I/Xba I digestion of the miRNA candidates in the pENTR/pSM2 (CMV/TO) vector. The empty vector will hava a 154 bp band (lane 9) where as the vectors with the insert will have a 262 bp band (lanes 1-8).

6. Send the positive clone(s) for sequencing using either the pENTRforward or pENTRreverse primer. A typical result obtained with the pENTRreverse primer is shown in Figure 14:

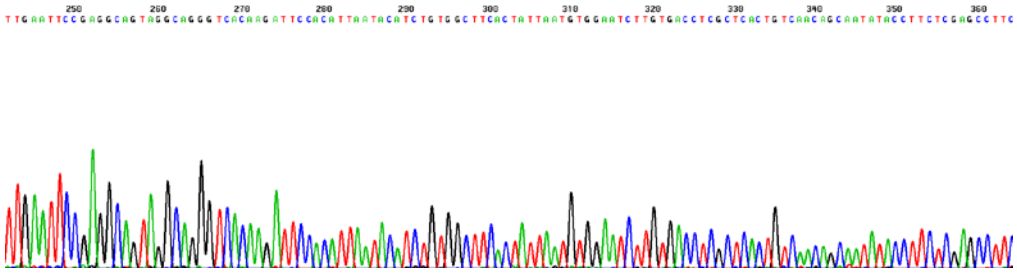


Figure 14. Sequencing of the pENTR/pSM2 (CMV) Asf1a clone with pENTRrev. The sequence shown encompasses the Eco RI and Xho I cloning sites (gaattc and ctcgag respectively) This clone was used to deplete Asf1a from U2OS cells ([7]).

NOTE: The TA cloning step is not necessary to clone miRNAs and the PCR product can be digested with Xho I/Eco RI and directly cloned into the pENTR/pSM2 vector of choice. I prefer to go through TA cloning because it gives me a higher cloning efficiency.

2.5- Cloning miRNAs from the Open Biosystems libraries into the pENTR/pSM2 Series.

Since the pSM2c plasmid was used as the basis for the Open Biosystems retroviral library and that the same library was transferred into the pGIPZ lentiviral backbone, there might be some interest to subclone directly clones obtained from these libraries into the miRNA-based pSM2 Series Entry vectors of this system. Furthermore, the pSM2 Series has the option to generate inducible miRNA that the pSM2c and pGIPZ libraries don't. Here are some suggested "cut and paste" digestion that can be done to transfer miRNAs from these libraries into our Entry vectors. Please note that we did not test these combinations and, as of August 2009, it is unknown if there might be unforeseen problems. Also, make sure that your miRNA sequence does not have one of the enzyme recognition sequence used for the cut and paste. These digestions transfer also the 5'miR30 and 3'miR30 sequences.

A- Subclone the entire U6-MiR5'-sequence-MiR3' cassette into pENTR1A

Digest the pSM2 plasmid with Bam HI and Pme I and ligate into pENTR1A previously digested with Bam HI and Eco RV.

B- Subclone the MiR5'-sequence-MiR3' into pENTR/pSM2(CMV/TO, w55-1)

Digest the pSM2 plasmid with Spe I or Sal I and Mfe I and ligate into plasmid w55-1 previously digested with either Spe I or Sal I and Mfe I. Alternatively, digest the pSM2 vector with Spe I or Sal I and Pme I and ligate into plasmid w55-1 previously digested with either Spe I or Sal I and Xba I that was made blunt with either a mung bean nuclease treatment or Klenow polymerase.

C- Subclone the MiR5'-sequence-MiR3' into pENTR/pSM2(CMV, w57-1)

Digest the pSM2 plasmid with Sal I and Mfe I and ligate into plasmid w57-1 previously digested with Sal I and Mfe I. Similar to B, the pSM2 plasmid can be digested with Sal I and Pme I and ligated into an Sal I/Xba I (blunt) w57-1 plasmid.

D- Subclone the MiR5'-sequence-MiR3' into pENTR/pSM2(CMV) GFP (w513-1)

Digest the pSM2 plasmid with Sal I and Mfe I and subclone into the w513-1 plasmid digested with Sal I and Mfe I. Unlike B and C, the Xba I site is not unique anymore after the insertion of the GFP cDNA.

3- LR Recombination into the Destination vector

Introduction After you have generated an entry clone, you are ready to perform the LR recombination reaction using the Entry construct and a suitable Destination vector. Three types of Lentiviral Destination vectors can be used: the pLenti X1 Series, the pLenti X2 Series or the pLenti GFP Dest vector. For retroviruses, the pQCXIN X2 or pQCXIP X2 can be used. The different types of Destination vectors as well as their advantages/disadvantages are discussed in the introduction as well as in the reference article for the lentiviral system [7]. Also, the compatibles Destination vectors are shown in Table 1.

Materials Needed

- Minipreps of the Entry vector and the Destination vector
- LR Clonase enzyme mix and buffer (Invitrogen catalog no. 11791-019)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

1. Mix in a microcentrifuge tube:

TE Buffer (pH 8.0): **4 µl**
Entry clone containing the shRNA or miRNA: **1µl**
Destination vector: **1 µl**
5X LR Clonase Reaction Buffer: **2µl**
LR Clonase enzyme mix: **2 µl**

2. Incubate the reaction at room temperature for 3-18 hours.
3. Transform into **Stbl3** competent *E. coli* for the lentiviral vectors and **TOP10F'** for the retroviral vectors. Plate the transformation reaction on LB plates with ampicillin selection, **except for the pLenti X1 Zeo and pLenti X1 GFP-Zeo Destination vectors which require zeocin selection (50 µg/ml) in low-salt LB.**

NOTES:

The Invitrogen protocol recommends treating the LR reaction with proteinase K. I skip the treatment and it does not seem to affect the transformation reaction. Please consult the LR clonase instruction inset if you want to perform this step.

It is important to use an *E.coli* strain that will prevent unwanted recombinations. For lentiviruses, the Stbl3 strain gives the best results for stability and yields of plasmid. For retroviruses, TOP10F' gives better yields than Stbl3.

Colony picking and screening

1. Pick a colony and inoculate a 250 ml flask containing 50 ml of either low-salt LB with 50 μg/ml Zeocin or LB and ampicillin. The LR reaction is usually very efficient and requires very few colonies to be picked. I usually pick 1-2 colonies. Grow the colonies overnight at 37°C in a shaking incubator.
2. Take a small aliquot of the overnight culture and do a miniprep. We normally use the Zippy kit from Zymo Research and use 600 μl of the overnight culture. Keep the remaining of the culture at 4°C. It will be used later to isolate plasmid DNA for the transfection step (see below).
3. Confirm the identity of the clone by restriction digest. I normally choose a restriction enzyme that cuts 4-6 times the vector backbone to generate a ladder of different size DNA. This confirms that the recombination took place and that no other rearrangement of the backbone occurred. In the rare instances where the clone was not good, obvious rearrangements occurred and were easily detected by the restriction digest. I usually digest with Eco RV or Nco I. For example, Eco RV digestion of a pENTR/pTER shRNA recombined into the pLenti X2 Puro DEST vector gave the pattern shown in Figure 15:

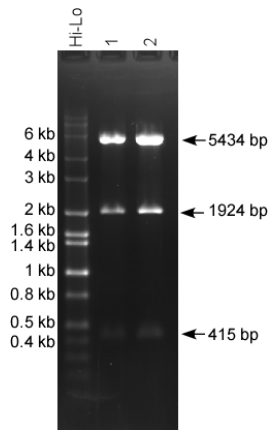


Figure 15. Digestion of pLenti X2 Puro/pTER shRNA with Eco RV. Two clones encoding different shRNAs in the pLenti X2 Puro Destination vector were digested with Eco RV and electrophoresed on a 0.8% agarose gel. Eco RV cuts four times in the vector generating products of 5434, 1924, 415 and 35 base pairs. The three biggest fragments are seen confirming that both clones are good.

Confirming the expression clone by sequencing

Sequencing the expression construct is not generally required. However, if you wish to sequence the H1/TO RNAi cassette, use the primer H1 Forward (5′-TGTTCTGGGAAATCACCATA-3′) for the shRNA constructs, the U6 primer (seq***) for the pSM2(U6)-derived vectors and the CMV forward primer (seq***) for the pSM2(CMV, CMV/TO)-derived vectors.

Purification of the plasmid DNA for transfection using the Qiagen Midi prep kit.

1. Take the remaining of the overnight culture and centrifuge the bacterial cells by centrifugation at 2500 rpm for 10 min at 4°C. Proceed to isolate plasmid DNA using one of the Qiagen Midi Isolation Kit (go to www.Qiagen.com for more details).
2. Resuspend the bacterial pellet in 4 ml Buffer P1.

3. Add 4 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min.
4. Add 4 ml of chilled Buffer P3 to the lysate, and mix by inverting 4–6 times.
5.Centrifuge at 2500 rpm for 10 minutes[1]. There should be a clear lysate with a bottom pellet and some debris floating on top of the lysate (see Figure 16 for an example).
6. Meanwhile, equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT and allow the column to empty by gravity flow. Add two layers of cheesecloth on top of the column.



Figure 16. E.coli lysate after centrifugation. There is a floating layer of cell debris, a cleared lysate and a pellet at the bottom of the tube. Re-centrifuge is the floating debris are not well separated as they might clog the column and decrease plasmid yields.

7. After the centrifugation, carefully decant the cell lysate onto the Qiagen tip-100 with the cheesecloth to make sure no debris enters the column. A successful decantation will leave the floating debris undisrupted on the side of the tube and the pellet at the bottom of the tube. An example is shown in Figure 17.



Figure 17. Successful decantation of the cleared lysate onto a Qiagen tip column. The circle of floating debris can be seen on the side of the tube and the pellet at the botton.

8. Allow the cleared lysate to enter the resin by gravity flow.
9. Wash the QIAGEN-tip with 2 x 10 ml of Buffer QC. Transfer the QIAGEN-tip over a 50 ml centrifuge tube.
10. Elute the plasmid DNA with 5 ml of Buffer QF.
11. Precipitate DNA by adding 3.5 ml of room-temperature isopropanol to the eluted DNA. Mix and centrifuge **immediately** at 6000 rpm for 45 min at 4°C. Carefully decant the supernatant.

12. Wash DNA pellet with 2 ml of room-temperature 70% ethanol and centrifuge at 1500 rpm for 5 min. Carefully decant the supernatant without disturbing the pellet.
13. Air-dry the pellet for 5–10 min, and redissolve the DNA in 100 μl of water.
14. Determine the DNA concentration by UV spectrophotometry

NOTES:

[1]The Qiagen manual does not suggest a centrifugation at this step for the Qiafilter prep version. However, the *E.coli* strain Stbl3 precipitates in very fine particles upon addition of buffer P3 and these particles can go through the Qiafilter and clog the Qiagen column. Follow the procedure for Qiafilter until the lysate is loaded on the Qiagen-tip 100 (step 6).

[2]Qiafilters are not obligatory. However, filtration of the lysate through cheesecloth is recommended to prevent the fine particles from entering and clogging the Qiagen column.

4A- Producing Lentiviruses in 293T Cells

Introduction To produce **lentiviruses**, three packaging plasmids will be co-transfected with the vector containing the shRNA/miRNA. Always use freshly thawed 293T cells that are not too confluent. Please read the appendix section on the 293T cell line since this cell line is peculiar and requires special care. Once the cells will be transfected, lentiviruses will be produced and released in the culture media. We will collect the viruses from the culture media at T=48 and 72 hours post-transfection, where the yields are the highest. We will centrifuge the culture media and filter the supernatant to remove cellular debris. We can also concentrate the lentiviruses by ultracentrifugation in step 5 if necessary.

Transfection procedure and virus production for a 10 cm dish

1. The day before the transfection, seed $5x10^6$ of freshly thawed[1] 293T cells.

2. Mix 15 µg of the transfer vector, 15 µg of pLP1, 6 µg of pLP2, 3 µg of pVSV-G in 1.5 ml of OPTI-MEM in one tube.

3. In another tube, mix 50 µl of Lipofectamine 2000 in 1.5 ml of OPTI-MEM.

4. Mix the DNA and the Lipofectamine 2000.

5. Incubate at room temperature for at least 25 minutes (up to 6 hours in theory).

6. Add to the DNA:Liposome complex (3.0 ml) to the dish already containing 7 ml of OPTI-MEM.

7. Incubate at 37°C overnight.

8. The next day, replace media with 10 ml of OPTI-MEM[2].

9. Collect viral supernatant at T=48h and T=72h post-transfection. Centrifuge supernatants and filter through a 0.2µM filter[3]. Store at -80°C. To obtain higher viral titers, viruses can be concentrated by ultracentrifugation (see below).

10. Determine viral titers by infecting HT1080 or HeLa cells.

NOTES:

[1]I usually thaw a fresh vial in a T-150 and wait 3-4 days until the cells are fairly confluent. Then I split them into 10cm dishes for the transfection. 293 cells should not be cultured for several passages as this will result in lower viral titers.

[2]D-MEM or other media can be used at this step. I prefer to use OPTI-MEM in case I need to concentrate the virus at a later stage. Ultracentrifugation of media containing serum has been shown to also precipitate more unwanted material than serum-free media. Serum-free D-MEM might be used, although I never tested that option.

[3]Some syringe filters have been shown to bind viruses and therefore reduce considerably viral titers. Use a low protein binding syringe filter. Also, several laboratories are using a 0.45μM filter instead of a 0.2μM. I did not notice any change in viral titers with the 0.2μM filters but because the transfection is done in the absence of antibiotics, I feel more safe to use a filter that can remove bacteria and fungi at the same time (of course, if there are noticeable bacteria/yeast in the dish, discard the dish, do not filter and keep the virus).

4B- Producing Retroviruses in 293GAG/POLCells

This protocol is for 10 cm or 15 cm dishes.

1. The day before the transfection, seed $5x10^6$, ($11x10^6$) of freshly thawed 293[1] GAG/POL cells.

2. Mix 12 µg (26µg) of the transfer vector, 1.2 µg (2.6 µg) of pGAG/POL, 1.2 µg (2.6 µg) of pVSV-G in 2.3 ml (5 ml) of OPTI-MEM in one tube.

3. In another tube, mix 50 µl (110 µl) of Lipofectamine 2000 in 2.3 ml (5 ml) of OPTI-MEM.

4. Mix the DNA and the Lipofectamine 2000.

5. Incubate at room temperature for at least 25 minutes (up to 6 hours in theory).

6. Add to the DNA:Liposome complex (4.6 ml) (10 ml) to the dish already containing 7 ml (15 ml) of OPTI-MEM.

7. Incubate at 37°C overnight.

8. The next day, replace media with 10 ml (20 ml) of OPTI-MEM[2].

9. Collect viral supernatant at T=48h and T=72h post-transfection. Centrifuge supernatants and filter through a 0.2µM filter[3]. Store at -80°C. To obtain higher viral titers, viruses can be concentrated by ultracentrifugation[4] (see below).

10. Determine viral titers by infecting HT1080 or HeLa cells.

NOTES:

[1]I usually thaw a fresh vial in a T-150 and wait 3-4 days until the cells are fairly confluent. Then I split them into 10cm dishes for the transfection. 293 cells should not be cultured for several passages as this will result in lower viral titers.

[2]D-MEM or other media can be used at this step. I prefer to use OPTI-MEM in case I need to concentrate the virus at a later stage. Ultracentrifugation of media containing serum has been shown to also precipitate more unwanted material than serum-free media. Serum-free D-MEM might be used, although I never tested that option.

[3]Some syringe filters have been shown to bind viruses and therefore reduce considerably viral titers. Use a low protein binding syringe filter. Also, several laboratories are using a 0.45µM filter instead of a 0.2µM. I did not notice any change in viral titers with the 0.2µM filters but because the transfection is done in the absence of antibiotics, I feel more safe to use a filter that can remove bacteria and fungi at the same time (of course, if there are noticeable bacteria/yeast in the dish, discard the dish, do not filter and keep the virus).

5-Concentrating the Lentivirus (optional)

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their transducibility. We normally concentrate the viruses using a two step ultracentrifugation procedure.

Materials to have on hand

Beckman Ultracentrifuge
Beckman rotors SW28 and SW55
Beckman polyallomer centrifuge tubes 1x3.5 in (cat.#326823)
Beckman polyallomer centrifuge tubes 0.5x2 in. (cat.# 326819)
Hank's Balanced Salt Solution (Invitrogen cat.# 14025-092)

Ultracentrifugation procedure

1. Put the filtered supernatant from step 4 into a centrifuge tube for the SW28 rotor (cat.#326823).
2. Centrifuge at 20,000 rpm for 90 minutes[1].
3. Carefully remove ~90% of the supernatant (~16 ml) and resuspend the pellet by adding ~2 ml of Hank's balanced salt solution (HBS, final volume ~ 4 ml).
4. Put in a centrifuge tube for the SW55 rotor (cat.#326819) and centrifuge at 21,000 rpm for 2 hours.
5. Carefully remove the supernatant and resuspend the pellet in ~ 50 μl of HBS.
6. Incubate with shaking for one hour at 4^{o}C.
7. If desired, determine the virus titer (see appendix) or aliquot and store at -80^{o}C until use. Alternatively, virus concentration can be determined by ELISA using an antibody against the p24 viral capsid protein.

NOTE:

[1]For some applications, a single ultracentrifugation will concentrate the virus at a desirable level. Depending on our experiment, we do either a single centrifugation and resuspend in 2-4 ml of HBS (for a 5-10 fold concentration) or a dual centrifugation and resuspend in 50 μl of HBS (for a 500-1000 fold concentration).

Long-term storage

Place lentiviral stocks at -80°C for long-term storage. Repeated freezing and thawing is not recommended as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, we recommend re-titering your viral stocks before transducing your mammalian cell line of interest.

6- Transduction and expression of the shRNA/miRNA

Multiplicity of infection (MOI)

Before transducing a cell line with a virus, titering of the virus should be done to ensure that similar levels of control and experimental viruses are transduced. A protocol to determine viral titers is included in the appendix. Also, we recommend to transduce cells at a relatively low MOI (<5) to reduce the risk of insertional mutagenesis and stress to the cell. This is particularly important in the case of primary cells whereby transduction of viruses at high MOI might result in senescence or apoptosis. In the case of inducible expression of shRNAs/miRNAs, transduction at low MOI of the T-REx cell line is critical to ensure that sufficient levels of the Tet repressor can occupy all the Tet operators in order to prevent leakiness of the shRNA/miRNA.

Clonal isolates or population?

Depending on the efficiency of the shRNA/miRNA and of the T-REx cell line, it might be necessary to isolate colonies in order to obtain maximal depletion of the target protein. It is recommended to freeze-down aliquots of the cells as early as feasible, especially if depletion of the target protein is deleterious to the cell. In this case, subpopulations with lower levels of shRNA/miRNA might have a growth advantage and slowly take over the culture. This difference in shRNA/miRNA levels could be due to either the number of viruses integrated in the host genome, the genomic location of the viral insertion (in active versus inactive chromatin), or methylation of the promoter driving the shRNA/miRNA expression. For inducible expression of an shRNA/miRNA or cDNA that is detrimental to the cell, leaky cells should be eliminated from the population and a relatively unleaky population should result, as we have shown for the dominant-negative protein iTIN2-15C [7].

Time for optimal depletion.

It is recommended to do a time-course for the depletion of the protein of interest to determine the time for optimal depletion. Several factors will affect depletion efficiencies: the efficiency of the shRNA/miRNA, the stability of the protein, the transcription levels/mRNA levels to be depleted, and the response of the cell to the loss of the protein. If the protein has a short half-life, it might be depleted in less than 24 hours, but if it has a long half-life, it could take 72-96 hours to reach the maximal depletion levels. Also, if the protein is critical for the cell survival or growth, its transcription levels might be increased to counteract the shRNA. Alternatively, the promoter driving the shRNA/miRNA expression might get methylated and at longer time points, the protein levels might increase. If rapid depletion of the protein is required for the experimental setup, an alternative is to use the FKBP destabilization domain system [75,76] also commercialized under the name ProteoTuner (Clontech) in conjunction with a constitutive shRNA. In this case, the FKBP-fused protein should contain mutations in its cDNA to confer resistance to the shRNA. We generated Entry vectors to allow FKBP fusions to cDNAs. Using this system, proteins can be degraded in 1-3 hours, in cycling or mitotically-arrested cells [7]. Please refer to the manual on expression studies for more details.

Other factors
There are several other factors that might affect the outcome of the experiment. For example, different cell line can have different transduction efficiencies. When working with a new cell line, it is always a good control to transduce a virus expressing GFP and monitor transduction efficiencies by counting the percentage of fluorescent cells. The amount of doxycycline used to induce depletion can vary in different cell line. Furthermore, some cell line like U2OS have been shown to undergo apoptosis at doxycycline concentrations of 5.0 ug/ml and slowed growth at 2.0 ug/ml [77].

Tetracycline in fetal bovine serum
Some lots of fetal bovine serum (FBS) might contain traces of tetracycline since this antibiotic is often used in farm animals to prevent diseases. If cells are grown in medium containing FBS that is not reduced in tetracycline, you may observe some basal expression of your shRNA (as measured by target gene knockdown) in the absence of doxycycline. It is recommended to use a "Tet-System approved " FBS (Clontech) or "Tet-deprived" FBS (Hyclone) or a "Tet-Tested" FBS (Atlanta Biologicals).

Negative controls for RNAi experiments: As mentioned in the introduction of this manual, expression of shRNAs/miRNAs might sequester some essential components of the RNAi machinery and have secondary effects. The resulting phenotype would not be due to the depletion of the target protein, but to the lack of a functional RNAi machinery. To control for such an effect, we recommend using an shRNA against either the firefly luciferase (LUC) or GFP in the same vector backbone as the one used for the experimental shRNA. Some Entry vectors and recombined Destination vectors were generated for both shLUC and shGFP and sent to Addgene. Their maps can be found in the appendix.

Materials required
• A viral stock of the shRNA/miRNA
• A host cell line (T-REx for inducible depletion)
• 6 mg/ml Polybrene® (Sigma H-9268)
• 1 mg/ml doxycycline (for inducible depletion)
• Tissue culture plates or dishes
• Antibiotic (blasticidin, hygromycin, neomycin, puromycin or zeocin) for selection.

Transduction procedure (one well of a six-well plate)
Note: These conditions worked with most cell lines I tested. There might be some adjustments required for some cell lines. It is recommended to test the conditions with a GFP-expressing virus to monitor virus production and transduction.

1. The day before the transduction, plate 100,000 cells in each well of the six-well plate. For 10 cm dishes, I normally use 800,000 cells and for 15 cm dishes, I use 2 million cells. The cells should be between 40-50% confluent at the time of transduction.

2. I normally start my transductions between 16:00-20:00 (4pm-8pm) until 10:00-12:00am the next day. Depending on the cell type, longer incubation might be needed for better transduction efficiencies or shorter incubations to decrease the toxicity of the virus/polybrene. I normally do my infection at a MOI between 0.2-0.6[1]in a final volume of 950 ul with 6 ug/ml polybrene.
3. Incubate overnight in the incubator.
4. Remove the media and rinse twice with PBS. Add fresh media (2 ml per well for six-well plates, 10 ml for 10 cm dishes and 20-25 ml for 15 cm dishes).
5. The next day (48h post-transduction), add selection to kill untransduced cells[1].
6. If working with a cell line with an unlimited life-span, you can pick colonies and expand each clone. Alternatively, if working with primary cells, you may pool the heterogeneous population of resistant cells. In our hands, both approaches work.

7a. <u>For inducible depletion:</u> Expand the cells until there are enough cells to freeze. Inoculate two wells of a six-well plate to test the inducibility of the depletion. The next day, add doxycycline to one well at a final concentration of 1 ug/ml and incubate for 48h. After 48h, change doxycycline and re-incubate for another 48h[3].

7b. <u>For constitutive depletion:</u> At 48-96h post-transduction, collect cells and analyze for protein depletion[3].

Notes

[1] I normally get viral titers around 10^5 colony forming unit (cfu) per ml of viral supernatant. Therefore, for a viral titer of $1x10^5$ cfu/ml, I would use 200 ul of virus to obtain a MOI of 0.2. Add media to a final volume of 950 ul and polybrene at a final concentration of 6 ug/ml. The final volume must be kept as low as possible to maximize contact between the viruses and the cell. For 10 cm dishes, the final volume is 5 ml and for 15 cm dishes, the final volume is 12 ml.

[2] The drug for the selection will depend on the Destination vector used during the LR recombination reaction step. The concentration to be used will vary amongst cell lines. A kill curve might be useful or a survey of the literature might serve as a good starting point. Alternatively, the use of a GFP-expressing virus encoding the drug resistance of choice would allow to determine if most untransduced cells are killed by the drug selection and also if some GFP-expressing cells could be killed by the use of a drug at a too high concentration.

[3] Depending on the target protein, shorter incubations could generate a good depletion. A depletion time-course should be done once it is determined that an shRNA/miRNA can deplete the protein of interest.

For some examples of RNAi depletion using either shRNAs or miRNAs, please refer to the article describing these vectors [7].

Appendix

1) Recipes

LB (Luria-Bertani) Medium (1 Liter)
10 g Tryptone
5 g Yeast Extract
10 g NaCl
pH 7.0

1. Dissolve the tryptone, yeast extract, and NaCl in 950 ml of deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle.

NOTE : We normally prepare 5X LB that we dilute in water.

Low Salt LB Medium for Zeocin selection
10 g Tryptone
5 g Yeast Extract
5 g NaCl
pH 7.5

1. Prepare as above, except use 5g of NaCl per liter and adjust the pH to 7.5. For plates, add 15 g/L agar before autoclaving.
2. Autoclave and let the solution cool to ~55°C before adding the Zeocin to a final concentration of 50 µg/ml.
3. Store at +4°C in the dark. Plates containing Zeocin™ are stable for 1-2 weeks.

2) Genotypes of the *E.coli* strains

TOP10F' (Invitrogen)
Genotype: F´{*lac*I$_q$ Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1*ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G. **Description:** This *E.coli* strain is very useful for general cloning and gives lower recombinations than the DH5α strain, especially for the cloning of shRNAs.

DB3.1 (Invitrogen)
Genotype: F$^-$ *gyr*A462 *end*A1 *gln*V44 Δ(sr1-recA) *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 Δ*leu mtl*-1. **Description:** This strain is used to propagate plasmids containing the ccdB gene which is toxic to strains not containing the *gyr*A462 gene.

Stbl3 (Invitrogen)
Genotype: F$^-$ *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ$^-$ *leu mtl*-1. The strain is *end*A1+ **Description:** This strain is derived from the HB101 strain and is useful for the cloning of unstable DNA and repeats found in the LTRs of the lentiviral backbone.

3) 293T and 293GAG/POL cell lines

First, the health of the 293 packaging cell line is critical. If the cells are too confluent or not grown under optimal conditions, it will result in lower viral titers. Similarly, if the cells have been passaged for a long time, it will also yield lower titers. We always thaw a fresh vial of 293 cells when we are producing viruses. Second, the transfection procedure, liposome reagent, the quality of the media used as well as the amounts and quality of the vectors co-transfected are also critical. Third, the viral titers will also be proportional to the volume of media used to grow the cells in order to collect the viral supernatant. Finally, the cell line used for the titration, the amount of cells plated and the concentration of the selecting drug is also affecting viral titers. Other factors like the protein to be overexpressed or depleted might affect cell growth and viability and result in lower viral titers than expected. We recommend using a control vector expressing GFP to monitor transfection as well as transduction efficiencies. In addition, **293 cells are much less adherent than other cells and should be handled very gently when washing and changing medium**.

A special note about toxicity found in a 293T cell line. During the course of our studies, we noticed that one 293T cell line induced a change in U2OS morphology and induced senescence in primary WI38 fibroblasts (see http://ericcampeau.com/images/Toxicity%20mediated%20by%20some%20293T%20packaging%20cell%20lines.pdf) for more information). Although we did not identify the secreted factor responsible for this effect, the 293T cell line used to produce viruses should always be carefully monitored.

4) Titering of the viral stock

Because several factors can affect the production of viruses, it is recommended to measure titers each time a virus is produced. First, it will ensure that the transfection worked normally, and also that similar amounts of viruses transduced in each experiment. As viral titers decreases with long term storage at 4^{o}C or with each freeze/thaw cycles, it is also recommended to always establish viral titers between 72-96h post-transfection of the 293T cells. If the cells have been stored for more than two weeks at 4^{o}C or if they have been frozen and thawed several times, re-titering of the viral stocks is suggested.

Different cell lines are transduced with different efficiencies by viruses. Therefore, it is recommended to always use the same cell line when tittering viruses. We normally use HeLa, HT1080 and NIH 3T3 cells.

Procedure: Each virus will require a six-well plate. The wells will be transduced with various amounts of viruses in order to obtain colonies that can be easily counted. Then, the number of colonies counted will be adjusted for a volume of one milliliter to obtain the colony forming unit (cfu)/ml. For RNAi viruses, we typically obtain viral titers of 10^5 cfu/ml.

Material required

- A viral stock of the shRNA/miRNA
- HeLa or HT1080 cells
- 6 mg/ml Polybrene® (Sigma H-9268)
- 6-well tissue culture plates
- Antibiotic (blasticidin, hygromycin, neomycin, puromycin or zeocin) for selection.

1. The day before the transduction, plate 25,000 cells in each well of a six well plate. Use one six-well plate for each virus to be titered.
2. On the day of the transduction, add the following amounts of media containing polybrene at a final concentration of 6 ug/ml to six eppendorf tubes: 850 ul, 940 ul (2 tubes), 949 ul (2 tubes) and 950 ul as shown in Figure 18.

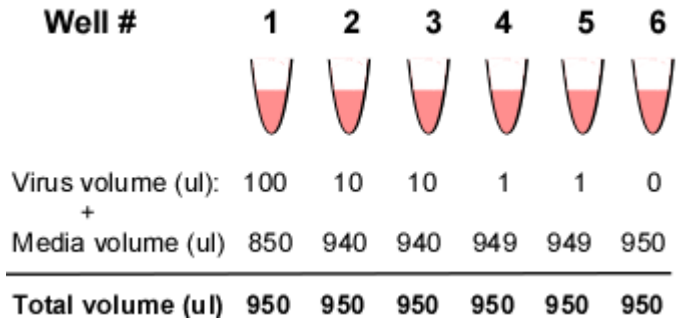


Figure 18. Titering of the viral stock in a six-well plate. Different amounts of viral supernatants are added in tubes 1-6. Media containing polybrene (6ug/ml) is added to each tube for a final volume of 950ul.

3. Add 100 ul, 10 ul (2 tubes), 1 ul (2 tubes) to each tube respectively to end up with a total volume of 950 ul.
4. Transfer the content of each tube into a well of the six-well plate as shown in Figure 19. Incubate overnight in the incubator.

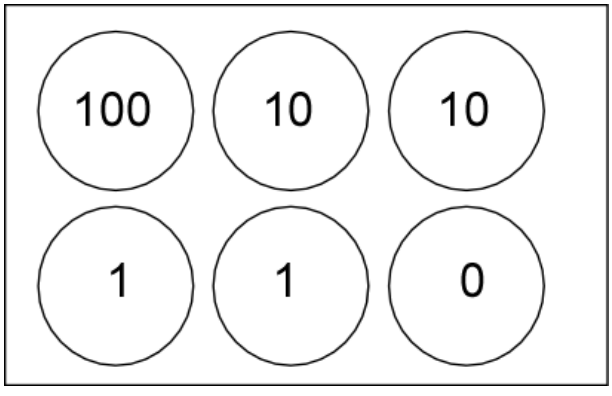


Figure 19. Layout of the viral titering plate. For each well, the number represents the amount of viral supernatant originally added to each tube.

5. The next day, remove the media/virus, wash twice with PBS and add fresh media.
6. The next day (48h post-transduction), add selection and put back in the incubator.
7. Wait until colonies can be clearly seen with the naked eye. Monitor to make sure the cells are not dying or senescing due to the depletion of the protein of interest.
8. Stain with crystal violet (see below for protocol).

Note: Titering viruses is a useful way of measuring the amounts of live and functional viruses present after transfection of the 293T cells. However, **these titers will not be accurate if the protein to be depleted is essential for the cell.** Using a T-REx version of the cell line could be a way to obtain more accurate titers. Alternatively, an ELISA assay could be done against the p24 viral capsid protein. In this case however, the amounts will represent the total amount of viruses, including live, dead and non-functional viruses.

5) Crystal Violet Staining and calculation of viral titers

* This protocol was adapted from whitelabs.org
(http://www.whitelabs.org/Lab%20Protocols/Mammalian%20Cell%20culture%20and%20manipulations/Crystal%20Violet%20Staining.htm)

1. Place plates on ice.
2. Wash two times with 1X PBS.
3. Fix cells with ice-cold methanol (stored at $-20^{o}C$) for 20 minutes.
4. Aspirate methanol from plates, move off the ice and add enough 0.5% crystal violet solution (made in 25% methanol and stored at room temperature) to cover bottom of plate.
5. Incubate at room temperature for 20 minutes.
6. Pour off crystal violet solution into a beaker (can re-use).
7. Carefully rinse plates in ddH_20 (normally, dunk twice in cold water).
8. Allow plates to dry at room temperature (may take overnight).

A typical result is shown in Figure 20 for the pQCXIN X2/pTER shLUC plasmid (w347-1, Addgene #17489).

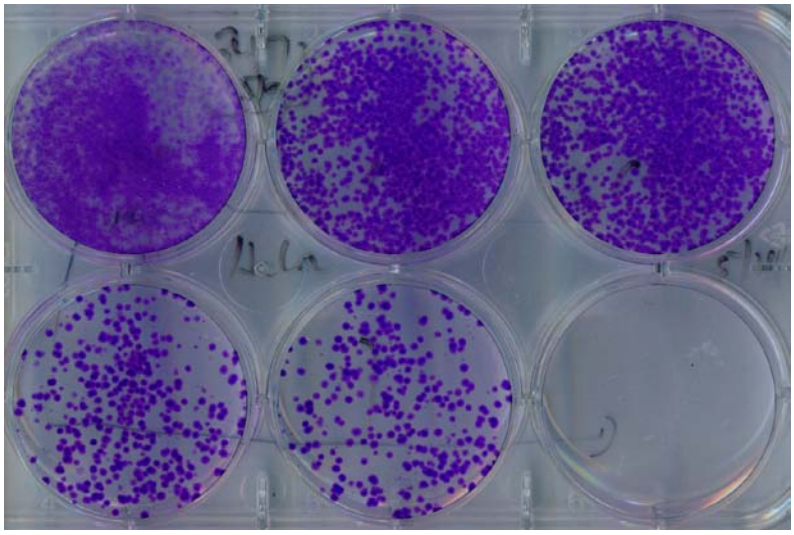


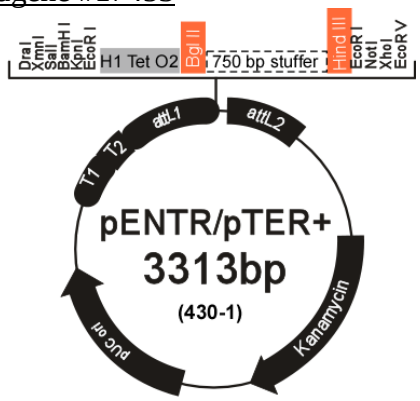
Figure 20. Titration of the pQCXIN X2/pTER shLUC plasmid. The pENTR/pTER shLUC (w177-1, Addgene #17472) was recombined with the pQCXIN X2 DEST vector (w310-1, Addgene #17399) to generate the pQCXIN X2/pTER shLUC plasmid. The plasmid was transfected in 293Gag-pol cells with the pGAG-pol and pVSV-G packaging vectors and viral supernatant were titrated using HeLa cells.

In the above example, 341 colonies were counted for well #4 and 283 for well #5. Therefore, the average is 312 colonies. In these wells, 1 ul of viral supernatant was added. So to obtain the titer per ml of virus, we multiply by 1000 to obtain a titer of **3.1×10^5 cfu/ml**. Please note that it is probably an underestimation as some colonies were overlapping with each other. A more accurate titration would require the use of 0.1 ul instead of 1 ul of viral supernatant.

6) Maps and features of vectors for RNAi studies

NOTE: The full sequence and the maps of the vectors are also available on the PLoS ONE web site at http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0006529 and at http://ericcampeau.com.

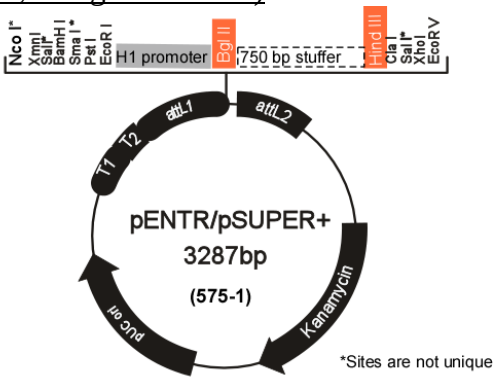
A) pENTR/pTER+ (430-1), Addgene #17453



Comment s for pENTR/pTER+
3313 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
H1 RNA promoter: bases 494-715
750 bp stuffer: bases 716-1472
attL2: bases 1542-1640
Kanamycin resistance gene: bases 1764-2573
pUC origin: bases 2635-3308

B) pENTR/pSUPER+ (575-1, Addgene #17338)

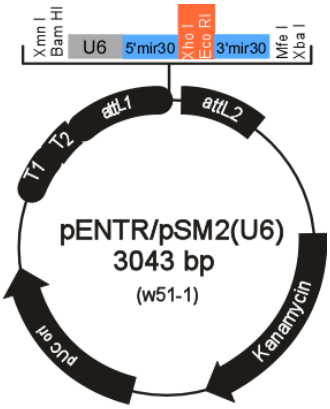


Comment s for pENTR/pSUPER+
3313 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
H1 RNA promoter: bases 494-715
750 bp stuffer: bases 716-1472
attL2: bases 1542-1640
Kanamycin resistance gene: bases 1764-2573
pUC origin: bases 2635-3284

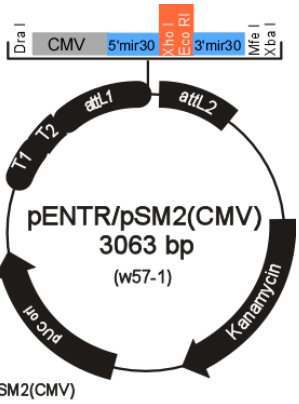
Note: pENTR/pTER+ can be used for inducible expression of shRNA only when expressed in a T-REX cell line. It will be constitutive in other cell lines. The pENTR/pSUPER+ vector can be used for constitutive expression of shRNA in any cell line.

C) pENTR/pSM2(U6) (w51-1, Addgene #17387)



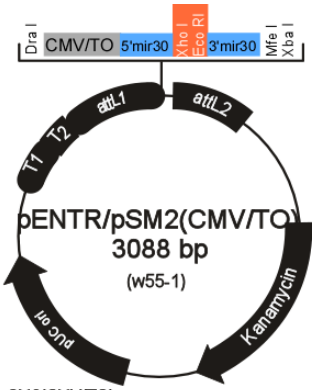
Comment s for pENTR/pSM2(U6)
3043 nucleotides
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
U6 RNA promoter: bases 488-989
5'miR30 context: 999-1112
shRNA cloning sites (Xho I/Eco RI):1113-1130
3'miR30 context: 1131-1237
Pol III transcription termination: 1238-1243
attL2: bases 1272-1372
Kanamycin resistance gene: bases 1494-2303
pUC origin: bases 2368-3040

D) pENTR/pSM2(CMV) (w57-1, Addgene #17389)



Comment s for pENTR/pSM2(CMV)
3063 nucleotides
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
CMV promoter: bases 474-1013
5'miR30 context: 1014-1132
shRNA cloning sites (Xho I/Eco RI):1133-1150
3'miR30 context: 1151-1253
attL2: bases 1292-1392
Kanamycin resistance gene: bases 1514-2335
pUC origin: bases 2407-3060

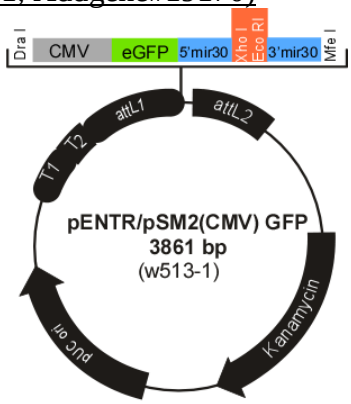
E) pENTR/pSM2(CMV/TO) (w55-1, Addgene#17388)



Comment s for pENTR/pSM2(CMV/TO)
3088 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
CMV/TO promoter: bases 474-1035
5'miR30 context: 1039-1157
shRNA cloning sites (Xho I/Eco RI):1158-1175
3'miR30 context: 1176-1273
attL2: bases 1317-1417
Kanamycin resistance gene: bases 1539-2361
pUC origin: bases 2431-3086

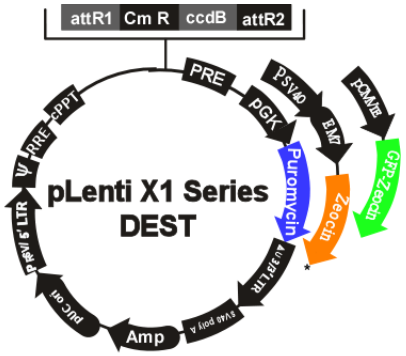
F) pENTR/pSM2(CMV) GFP (w513-1, Addgene#19170)



Comments for pENTR/pSM2(CMV) GFP
3861 nucleotides

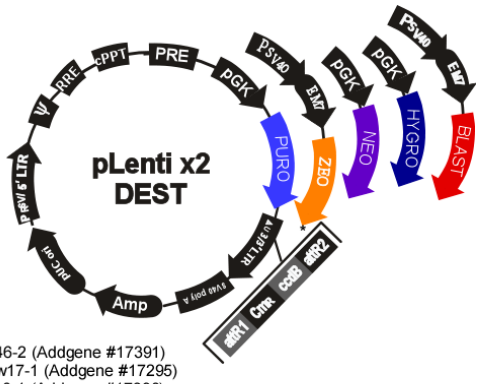
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
CMV promoter: bases 474-1013
Enhanced green fluorescent protein (eGFP): bases 1083-1802
5'miR30 context: 1813-1930
shRNA cloning sites (Xho I/Eco RI):1931-1948
3'miR30 context: 1949-2051
attL2: bases 2090-2190
Kanamycin resistance gene: bases 2312-3135
pUC origin: bases 3204-3859

G) pLenti X1 Series



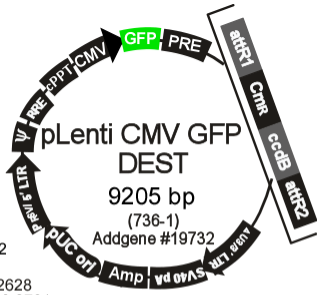
CMVIE-GFP-Zeocin: 720-2 (Addgene #19277)
Puromycin: 694-6 (Addgene #17297)
Zeocin: 668-1 (Addgene #17299)

H) pLenti X2 Series



Blasticidin: w46-2 (Addgene #17391)
Hygromycin: w17-1 (Addgene #17295)
Neomycin: w18-1 (Addgene #17390)
Puromycin: w16-1 (Addgene #17296)
Zeocin: 749-3 (Addgene #21562)

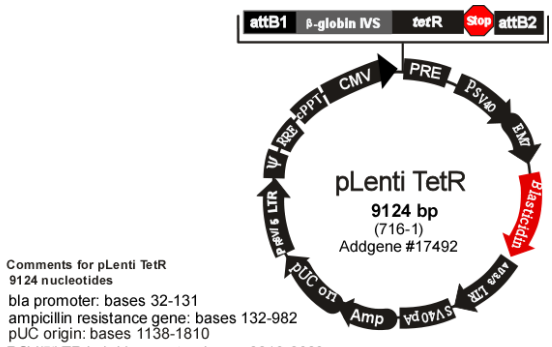
I) pLenti CMV GFP DEST (736-1, Addgene #19732)



Comments for pLenti GFP DEST
9205 nucleotides
bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV1 Rev response element (RRE): bases 3272-3525
3 splice acceptor: base 3862
3 splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
CMV promoter: bases 4145-4718
Green Fluorescent Protein (GFP): bases 4755-5474
Woodchuck post-transcriptional element (PRE): bases 5493-6082
*att*R1 site: bases 6134-6259
Chloramphenicol resistance gene (Cm): bases 6368-7027
*ccd*B gene: bases 7368-7674
*att*R2 site: bases 7713-7840
ΔU3/3'LTR: bases 7945-8180
SV40 polyadenylation signal: bases 8250-8403

7) Other useful vectors submitted to Addgene:

A) pLenti CMV TetR Blast (716-1, Addgene #17492)

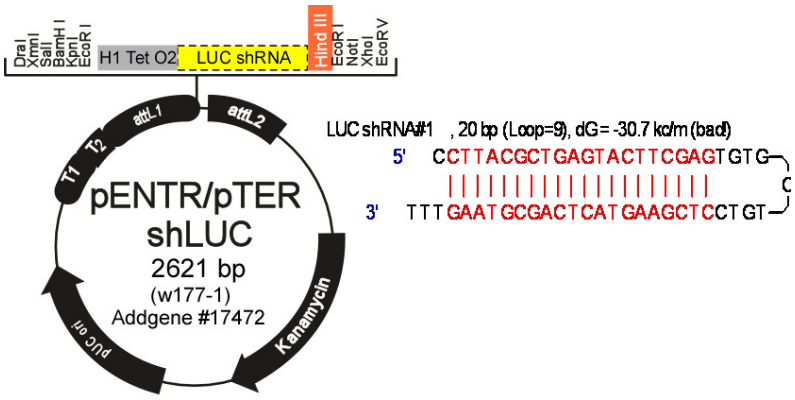


Comments for pLenti TetR
9124 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-982
pUC origin: bases 1138-1810
RSV/5'LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV1 Rev response element (RRE): bases 3272-3525
3 splice acceptor: base 3862
3 splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
CMV promoter: bases 4145-4730
attB1: bases 4764-4788
Rabbit β-globin intron II (IVS): bases 4933-5505
Tetracycline repressor (TetR) gene: bases 5589-6236
attB2: bases 6237-6261
Woodchuck post-transcriptional element (PRE): bases 6291-6884
SV40 early promoter and origin: bases 6932-7274
EM7 promoter: bases 7314-7379
Blasticidin resistance gene: bases 7380-7777
ΔU3/3'LTR: bases 7866-8098
SV40 polyadenylation signal: bases 8169-8322

This vector can be used to make T-REx cell lines. It has been used successfully in primary as well as transformed cell lines.

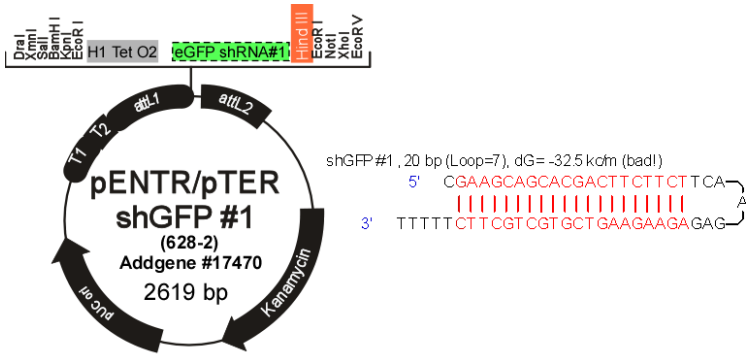
B) pENTR/pTER shLUC (w177-1, Addgene #17472)



Comment s for pENTR/pTER shLUC
2621 nucleotides

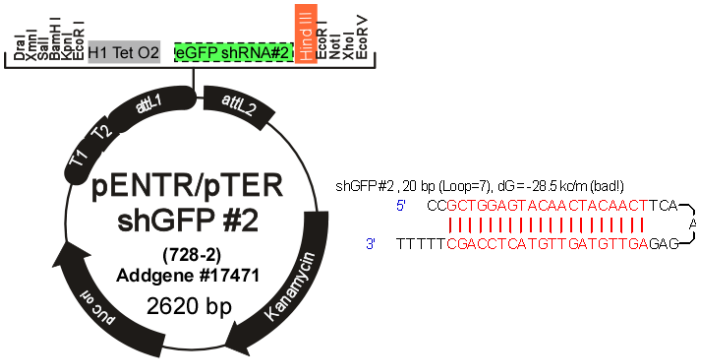
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
H1 RNA promoter: bases 494-715
Firefly luciferase shRNA: bases 722-778
attL2: bases 850-950
Kanamycin resistance gene: bases 1072-1881
pUC origin: bases 1991-2619

C) pENTR/pTER shEGFP#1 (628-2, Addgene #17470)



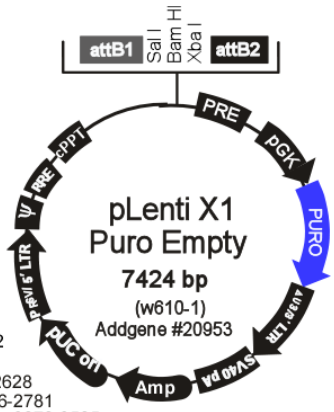
Comment s for pENTR/pTER shGFP #1
2619 nucleotides
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
H1 RNA promoter: bases 494-715
Enhanced GFP shRNA: bases 720-778
attL2: bases 848-948
Kanamycin resistance gene: bases 1070-1879
pUC origin: bases 1989-2617

D) pENTR/pTER shEGFP#2 (728-2, Addgene #17471)



Comment s for pENTR/pTER shGFP #2
2620 nucleotides
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
H1 RNA promoter: bases 494-715
Enhanced GFP shRNA: bases 721-779
attL2: bases 849-949
Kanamycin resistance gene: bases 1071-1880
pUC origin: bases 1990-2618

E) pLenti X1 Puro Empty (w610-1, Addgene #20953)

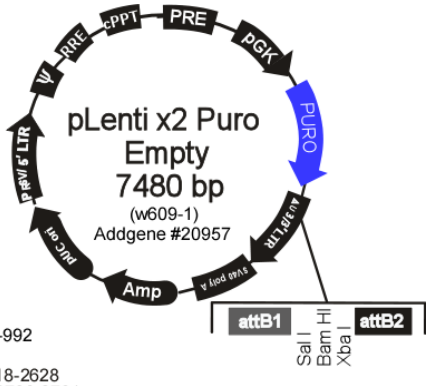


Comments for pLenti X1 Puro Empty
7424 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV-1 Rev response element (RRE): bases 3272-3525
3′ splice acceptor: base 3862
3′ splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
*att*B1 site: bases 4160-4185
Multiple cloning site: bases 4203-4251
*att*B2 site: bases 4251-4275
Woodchuck post-transcriptional element (PRE): bases 4305-4897
Murine phosphoglycerate kinase (pGK) promoter: bases 4901-5408
Puromycin resistance gene: bases 5428-6027
ΔU3/3′LTR: bases 6165-6398
SV40 polyadenylation signal: bases 6469-6622

Empty vector controls are useful to monitor possible stress or toxic effects associated with viral transduction and unrelated to the protein expressed or depleted.

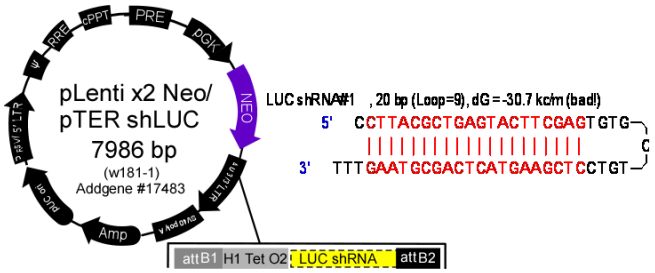
F) pLenti X2 Puro Empty (w609-1, Addgene #20957)



Comments for pLenti X2 Puro Empty RC
7480 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV-1 Rev response element (RRE): bases 3272-3525
3′ splice acceptor: base 3862
3′ splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
Woodchuck post-transcriptional element (PRE): bases 4182-4774
Murine phosphoglycerate kinase (pGK) promoter: bases 4780-5284
Puromycin resistance gene: bases 5305-5904
ΔU3/3′LTR: bases 6041-6454
*att*B2 site: bases 6109-6086
Multiple cloning site: bases 6152-6109
*att*B1 site: bases 6199-6175
SV40 polyadenylation signal: bases 6525-6678

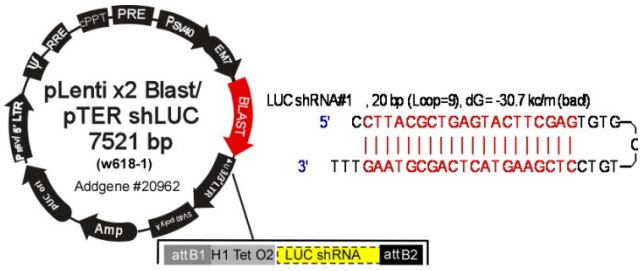
G) pLenti X2 Neo/pTER shLUC (w181-1, Addgene #17483)



Comments for pLenti X2/pTER shLUC Neo
7986 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV1 Rev response element (RRE): bases 3272-3525
3 splice acceptor: base 3862
3 splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
Woodchuck post-transcriptional element (PRE): bases 4182-4774
Murine phosphoglycerate kinase (pGK) promoter: bases 4805-5313
Neomycin resistance gene: bases 5327-6130
ΔU3/3'LTR: bases 6222-6959
attB2 site: bases 6264-6288
Firefly Luciferase (LUC) shRNA: bases 6358-6417
H1/TO promoter: bases 6424-6638
attB1 site: bases 6681-6705
SV40 polyadenylation signal: bases 7031-7184

H) pLenti X2/pTER shLUC BLAST (w618-1, Addgene #20962)



Comments for pLenti X2 Blast/pTER shLUC
7521 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV1 Rev response element (RRE): bases 3272-3525
3 splice acceptor: base 3862
3 splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
Woodchuck post-transcriptional element (PRE): bases 4182-4774
SV40 promoter: bases 4823-5164
EM7 promoter: bases 5205-5270
Blasticidin resistance gene: bases 5271-5669
ΔU3/3'LTR: bases 5690-6495
attB2 site: bases 5823-5799
Firefly Luciferase (LUC) shRNA: bases 5953-5904
H1/TO promoter: bases 6179-5954
attB1 site: bases 6240-6216
SV40 polyadenylation signal: bases 6566-6719

8) General geneclean protocol

- Migrate the digested plasmid at 80-90 volts until the band can be clearly seen and easily cut. Cut the band with a razor blade and determine its weight.

- Add 3X volumes of NaI and 1/10 volume of TBE modifyer (geneclean kit, Bio 101) if the agarose gel was run in TBE buffer (ex. 0.05 g band will need 150 ul of NaI and 15 ul of modifyer).

- Heat the tube at 45-55^{o}C to melt the agarose. Add 5-8 ul of glassmilk and incubate at room temperature for at least 5 minutes.(60 minutes maximum) Spin at maximum speed for 15 seconds.

- Remove supernatant and resuspend the pellet with 700 ul of cold New Wash. Spin at maximum speed for 15 seconds and remove supernatant. Repeat twice.

- After the last wash, remove as much of the New Wash solution as possible using a yellow tip. Let the pellet dry at room temperature (10 min to overnight).

- Resuspend the pellet in 7-20 ul of ddH_2O. Incubate at 50-55^{o}C for 2-3 minutes.

- Spin at maximum speed for 1 min. Keep supernatant and put it in the ligation tube.

9) TA cloning of PCR products protocol

- Purify the PCR product by geneclean as outlined above. However, elute in 6 ul of water instead of 20 ul.

- To the ligation tube, add: 6 ul of PCR product
  1 ul 10X ligation buffer
  2 ul pCR 2.1 vector
  1 ul of T4 DNA ligase.

- Incubate at 15^{o}C overnight.

- Transform in 50 ul of chemically competent *E.coli* TOP10F' using 4 ul of the ligation product. Plate on X-GAL/AMP (and/or KAN) plate and incubate at 37^{o}C overnight.

- Inoculate white colonies.

NOTE: If the PCR amplification was done using a polymerase having a 3'→5' exonuclease activity such as Pfu polymerase, you will need to add the A_s at the 3' end of the PCR product. At the end of the last PCR cycle, add dATP to a final concentration of 2mM (1 ul of 100 mM dATP in a 50 ul reaction) and 5 units of Taq polymerase (us. Gibco @ 5U/ul) and incubate at 72^{o}C for 20-30 minutes. Proceed with the agarose electrophoresis, geneclean and TA ligation as with the normal PCR products.

10) General plasmid subcloning.

- Inoculate colony or glycerol stock into 5 ml of LB broth and incubate with shaking overnight at 37^{o}C.
- Isolate plasmid using Wizard miniprep kit (Promega) or other procedure.
- Take 15 ul of the mini prep, add 5-10 units of restriction endonuclease and 1/10 volume of appropriate 10X restriction endonuclease buffer. Usually, I add 1 ul of enzyme and 1.8 ul 10X buffer.
- If the plasmid has to be digested with two enzymes, try to choose a buffer that will be compatible with both enzymes. Try buffer from different companies. If it is not possible, than try to choose a buffer that can be easily modified to the buffer of the second enzyme. For example, Gibco's React #2 can easily be changed to React #3 by adding 1 ul of 5M NaCl. If not, a phenol/chloroform, precipitation and resuspension will have to be done between the first and second enzyme.
- Incubate the plasmid with the buffer and the first enzyme for ~ 1 hour. Add the second enzyme and incubate another hour. Digestions can also be done overnight.
- Treat the plasmid into which the insert will be cloned with calf intestine alkaline phosphatase (CIAP). I usually add 2 ul of concentrated CIAP (Gibco's, ~ 26 U/ul) to the digested plasmid and incubate it at 37^{o}C for 15 min. I don't add the CIAP buffer supplied with the phosphatase.
- Load digested plasmids onto a 1% agarose gel and migrate at 80-90 volts until the bands can be easily cut.
- Cut bands with a razor blade, isolate the DNA with the Geneclean kit (**P6**), and resuspend pellet in 20 ul ddH_2O.
- Ligate the insert and the receiving plasmid as follows:

Tube 1: 12 ul insert	Tube 2: 8 ul insert
3 ul plasmid	6 ul plasmid
4 ul 5X T4 DNA ligase buffer	4 ul 5X T4 DNA ligase buffer
2 ul T4 DNA ligase (1 U/ul)	2 ul T4 DNA ligase (1U/ul)

Incubate the ligations at 15^{o}C overnight. Transform into *E.coli* using the protocol on the next page.

11) Transformation of chemically-competent *E.coli*

We normally use the Z-Competent *E.coli* transformation buffer set (catalogue # T3002) from Zymo Research to make our competent cells. We freeze the cells in 200-250 µl aliquots at -80°C.

- Thaw one vial of competent cells on ice (~5-10 minutes). Aliquot 50 µl of competent cells in an eppendorf tube.

- Add 2-5 ul of ligation reaction or up to 50 ng of plasmid.

- Incubate on ice 10-30 minutes.

- Heat shock at 42°C for 30 seconds. Put on ice for 30sec-2 minutes.

- Add 250 ul of SOC medium and transfer to a 10-15 ml tube.

- Incubate with shaking at 37°C for one hour.

- If blue/white selection is needed, spread 50 µl of a 20 mg/ml X-gal solution on the agar plates and leave it to dry at 37°C.

- Plate everything (ligation) or 25 µl (purified plasmid) on LB agar plates with appropriate antibiotic selection.

- Incubate at 37°C overnight.

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