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

Expression Studies

Version 1.0 March 2010

Disclaimer

This manual gives an overview of the various vectors that were made for overexpressions in mammalian cells. These vectors contain two genetic elements originally missing from the 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 [1,2,3,4]. I put together this manual by inserting the protocols I currently use to clone, express and produce lentiviruses and retroviruses as well as the genetic maps of the various vectors used for expression studies. Another manual dealing with vectors used for RNA interference studies is also available. 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. Additional improvements and 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 throughout this manual. Additional control vectors were also sent to Addgene, including empty vectors, vectors encoding the Firefly luciferase (LUC), the enhanced green fluorescent protein (GFP) or the β-galactosidase (LacZ).

An article describing the use of these vectors for expression and depletion studies was published in PLoS ONE [5]. 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, March 2010.

Overview

In recent years, overexpression of proteins in various cell lines has been an invaluable tool for cell biologists. However, with several companies providing different vectors using different technologies, it is often hard to find a system that offers the combined benefits of all of them. I therefore chose to build a viral vector system that could fit different needs for various experimental settings. I constructed retroviral and lentiviral vectors for either RNA interference (RNAi) or expression studies. This manual deals with vectors used for expression studies. Vectors for RNAi studies are covered in another manual available at http://ericcampeau.com/manuals. The viral vector system is based on Invitrogen's Gateway technology (link here) whereby the cDNA of interest is cloned into an Entry vector and then recombined with a viral Destination vector that will harbor the desired promoter and drug selection (Figure 1). This layout offers three main advantages over traditional plasmids used for expression in mammalian cells. First, the small size of the Entry vectors allow for easier cloning of cDNAs, compared to larger, more cumbersome mammalian expression vectors. Second, the LR recombination of the cDNA into the viral backbone is >95% efficient and significantly decreases unwanted cloning artifacts generated when working with viral backbones. Third, the wide collection of Entry and Destination vectors allows fusion of the protein of interest to a variety of tags for either detection, purification, degradation or post-translational modification of the protein under constitutive or inducible conditions and various drug selections.

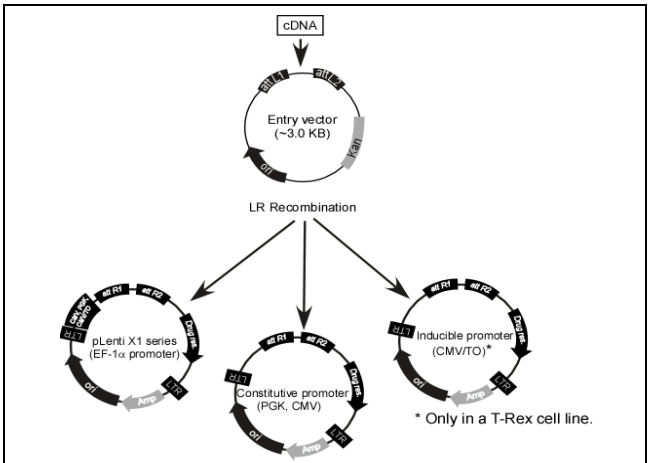


Figure 1. Overview of the Gateway technology to generate various viral vectors for expression studies.

The system includes:

- **Entry vectors with N-terminal epitope tags (V5, FLAG, GFP, DsRed2, GST)** to easily clone the cDNAs in frame to allow detection, purification of the protein of interest.

- **Entry vector with a C-terminal epitope tag (GFP)** in case a C-terminal tag is preferable for either protein stability or if the protein contains a signal peptide at its N-terminus.

- **Entry vectors with fusion proteins (NTAP, FKB12DD, Ubc9)** to either purify protein complexes by N-terminal affinity purification (NTAP), selectively degrade or induce the overexpressed protein (FKBP12DD), or detect potential sumoylation of the candidate protein (Ubc9).

- **Entry vectors for nuclear targeting of the protein (nuc-myc)** to target the protein of interest or one of its domain to the nucleus using the SV40 nuclear localization signal. The vector also has a C-terminal myc epitope for detection and/or purification.

• **Entry vectors with the Elongation Factor 1 alpha (EF1-α) promoter** to obtain physiological levels of expression of the target protein and prevent methylation of the promoter. These vectors also have a C-terminal His-V5 epitope tag.

Once the cDNA is inserted in the Entry vector, it can easily be recombined into one of the following Destination vector:

• **Destination vectors for constitutive expression under the CMV promoter (pLenti CMV series)**.

• **Destination vectors for constitutive expression under the PGK promoter (pLenti PGK series)**.

• **Destination vectors for inducible expression under the CMV promoter (pLenti CMV/TO series).** This system uses the T-REx technology from Invitrogen (link here).

• **Retroviral Destination vectors for either constitutive or inducible expression of the cDNA (pQCXI series).** For some applications, a retroviral backbone might be preferable. However, only the pQCXP CMV/TO DEST (w360-1) vector can be used for inducible expression of the cDNA and constitutive expression of the puromycin resistance gene. All the other vectors have the drug resistance gene after an internal ribosome entry site (IRES) which is regulated by the CMV/TO promoter. Therefore, both the cDNA and the drug resistance gene will be inducible in a T-REx cell line.

• **GFP fused to the Zeocin resistance gene (GFP-Zeo)**. Allows easy monitoring of the cells that have been transduced by immunofluorescence. The intensity of the GFP fluorescence is lower than the unfused GFP which might make it difficult to detect the GFP in live cells or in cell sorting experiments.

• **Destination vector expressing GFP (pLenti CMV GFP DEST)** to monitor depletion or sort cells through GFP fluorescence.

Precautions with lentiviruses and retroviruses

Biosafety Level 2

Lentiviruses and retroviruses 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 and retroviral 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 overexpress oncogenes (like RasV12) or cDNAs that inactivates tumor suppressor genes (like the SV40 Large T antigen, the papilloma E7 protein). 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/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Background

In this section, all the vectors are briefly introduced. In the following section, each vector will be explained in more details with their respective maps and multiple cloning sites (for Entry vectors).

i. General Entry vectors

A large variety of Entry vectors were generated to clone cDNAs encoding the protein of interest (POI) (Figure 2).

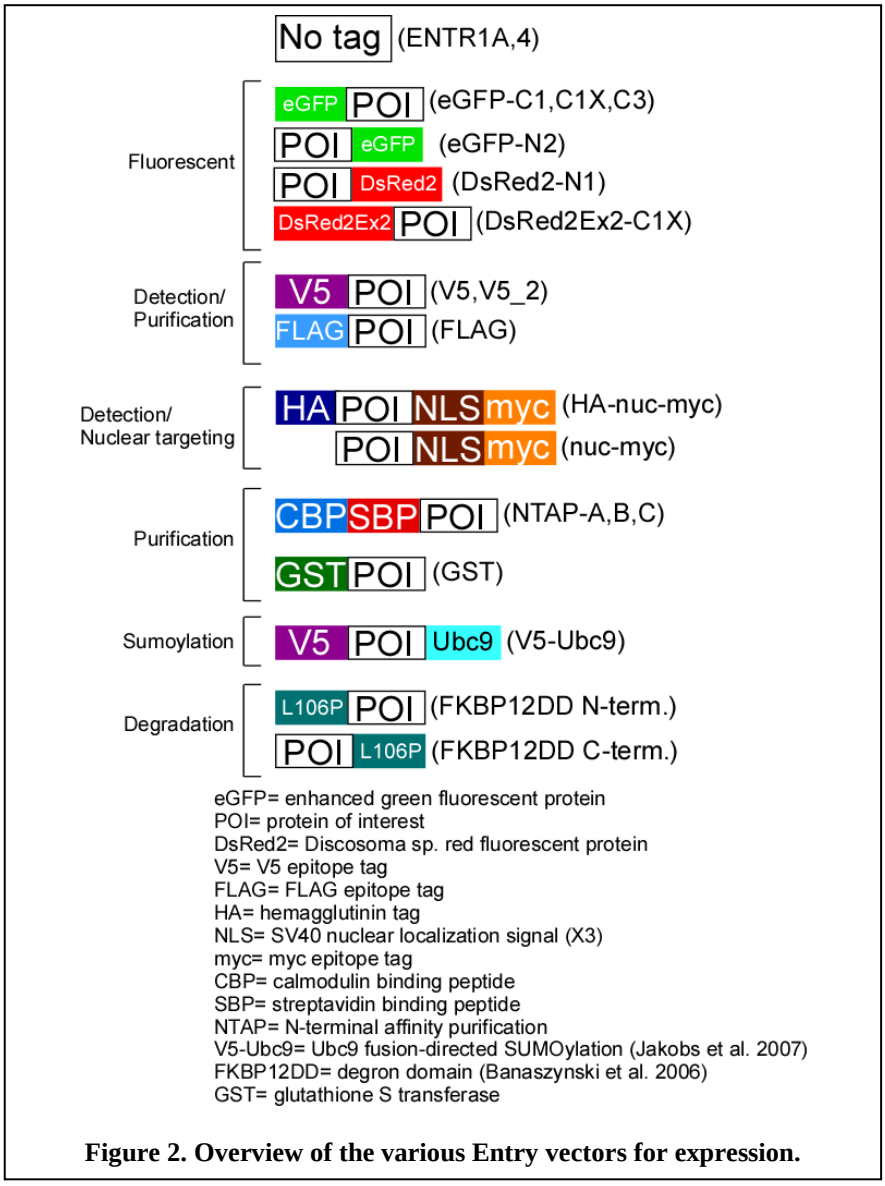


Figure 2. Overview of the various Entry vectors for expression.

The original pENTR1A and pENTR4 plasmids contain the ccdB killer gene [6]. To clone a cDNA into these vectors, two restriction endonucleases which cut on opposite sides of the killer gene have to be used in order to replace the ccdB sequence with the cDNA of interest and

thereby select against the parental plasmid. However, we removed the ccdB gene so that all of the multiple cloning sites could be exploited, generating constructs termed "pENTR1A no ccdB" and "pENTR4 no ccdB". We also generated a variety of Entry vectors that encode epitope tags at their N-terminus (GFP, V5, FLAG) or C-terminus (GFP, DsRed2, V5-His) in different reading frames to facilitate detection of the proteins of interest. For tandem affinity purification (TAP) experiments, we created Entry vectors encoding tandem N-terminal epitope tags in three reading frames, based on the pNTAP vector from Stratagene (NTAP,[7]). Additionally, we created an Entry vector encoding an N-terminal Glutathione-S-Transferase (GST) followed by a PreScission protease cleavage site (GE Healthcare Life Sciences), which can also be used for purification of the protein of interest via glutathione affinity supports. Other Entry vectors include either an N-terminal or C-terminal 12kDa FK506 Binding Protein (FKBP12) Destabilization Domain (DD) based on the Wandless lab design [8,9] (also commercialized by Clontech under the name ProteoTuner). This domain targets the protein of interest to the proteosome for rapid degradation upon removal of the protective ligand Shield-1 (Shld1), with faster kinetics than RNAi-mediated depletion [9]. For the pENTR-V5-Ubc9 plasmid, the SUMO E2 ligase Ubc9 was inserted at the C-terminus of the pENTR-V5 vector so that cDNAs could be inserted between the V5 epitope and Ubc9, generating a fusion protein that facilitates detection of the sumoylated target protein, thereby bypassing the requirement for an E3 SUMO ligase [10]. Finally, we made two vectors to target protein domains to the nucleus, the pENTR-HA-nuc-myc and pENTR-nuc-myc, which are derived from the pCMV/myc/nuc (Invitrogen). These vectors have three tandem copies of the SV40 Large T antigen nuclear targeting sequence [11]. Some vectors made by other laboratories are also included in this manual because they complement the collection of vectors we made. The origin of these vectors is indicated in the manual as well as on the web site.

ii. pEF-ENTR series

The cytomegalovirus (CMV) promoter is one of the most commonly used promoters to overexpress proteins. Several Destination vectors were generated to express proteins under the control of the CMV promoter (see below). However, the high levels of transgene expression attained by this promoter can sometimes be toxic to the cell. Alternatively, transgene expression can be lost due to methylation of the CMV promoter. For cases where the CMV promoter might not be optimal for cDNA expression, we also developed Entry vectors that contain the human EF-1α promoter (Figure 3). This promoter expresses the transgene at lower levels than the CMV promoter, which might be closer to the physiological conditions normally found in the cell. The EF-1α promoter has also been reported to be silenced at much lower levels *in vivo* compared to the CMV promoter [12,13,14]. These vectors encode a C-terminal V5-His epitope tag and were derived from the pEF-TRACER A, B, C plasmids (Invitrogen). One possible drawback of the EF-1α promoter is its large size (1.2 kb) compared to the PGK or CMV promoters (500-600 bp) which might result in lower viral titers for larger cDNAs. Also, the pEF-ENTR series can only be recombined with the pLenti X1 and pLenti CMV GFP series of Destination vectors (see Table 1 for more details on page 30).

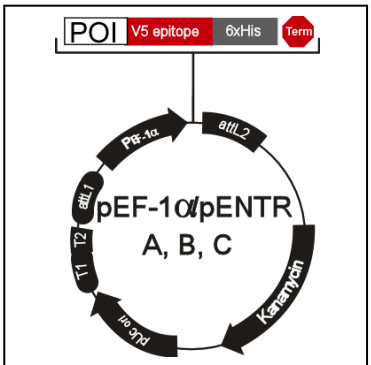


Figure 3. The pEF-ENTR series of Entry vectors.

iii. pLenti CMV, CMV/TO and PGK Destination series

We generated two series of Destination vectors for expression of cDNAs under the control of the CMV promoter and one series under the control of the PGK promoter (Figure 4). The pLenti CMV series has a constitutive CMV promoter and the pLenti CMV/TO series has an inducible promoter in T-Rex cell lines (see below). As mentioned previously, loss of protein expression *in vivo* due to promoter silencing can occur with the CMV promoter, but silencing is much less frequent for transgenes driven by the PGK and EF-1α promoters [12,13,14]. Therefore, we generated Destination vectors with the PGK promoter (Figure 4). The PGK promoter in the pLenti PGK series is smaller than the EF-1α promoter and therefore minimizes loss of viral titers when large cDNAs are inserted [15]. Also, the PGK promoter was inserted in Destination vectors and these vectors are consequently more versatile because they harbor more drug resistance genes, while the pEF-1α Entry vectors can only be recombined into the pLenti X1 Destination series (see Table 1 page 30 and Figure 7), which currently have only puromycin and zeocin resistance genes for selection. Please note that not all drug selections are available for all promoter combinations (Figure 4).

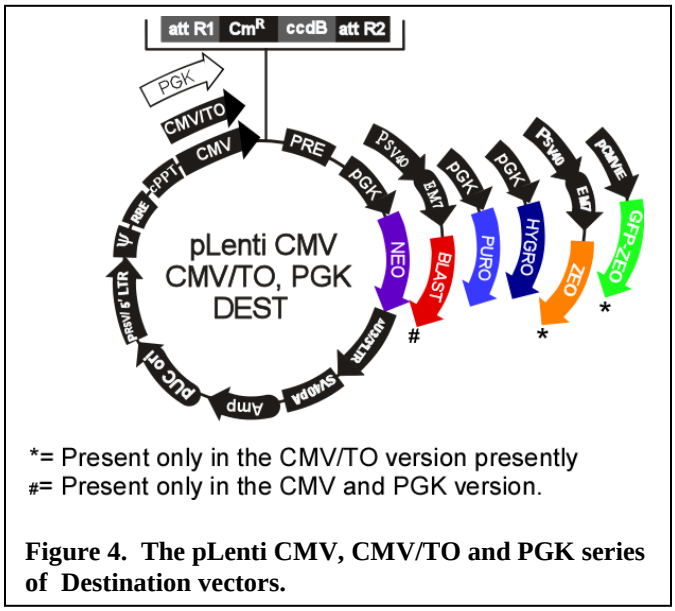


Figure 4. The pLenti CMV, CMV/TO and PGK series of Destination vectors.

Inducible expression using the T-Rex system

The pLenti CMV/TO series allows inducible transcription of the cDNA of interest upon addition of doxycycline in a T-REx cell line (Figure 5). We successfully used the T-Rex system to inducibly express several proteins. The availability of different drug selections and different constitutive and inducible promoters allowed us to create cell lines where either two proteins were simultaneously induced, or one protein was constitutively expressed and the other one was inducible.

There are several T-Rex cell lines available commercially through Invitrogen or made by other laboratories. It is also possible to create T-Rex cell lines by transfecting a plasmid expressing TetR (such as the pCDNA 6/TR plasmid available from Invitrogen) and selecting colonies that display a good inducibility. We also made a lentiviral vector to express TetR and easily make T-REex cell lines, the pLenti CMV TetR Blast vector (**716-1, Addgene #17492**). We used this vector to make lentiviruses to generate several T-REx cell lines in either primary fibroblasts or transformed cells [5]. 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 cDNA. 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

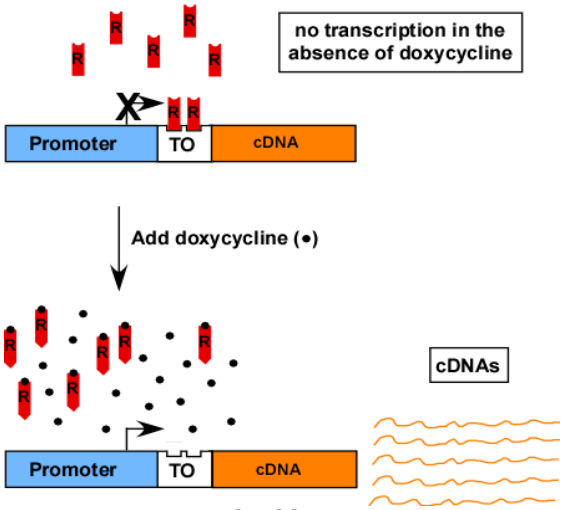


Figure 5. The T-Rex system to generate inducible protein expression. T-Rex cell lines express the tetracycline repressor (TetR, represented by R above). In the absence of tetracycline (or its derivative doxycycline), the repressor binds to the tetracycline operator (TO) to repress transciprion of the cDNA. Doxycycline induces a conformational change of the repressor so that it can no longer bind to the operator which results in derepression and transcription of the cDNA.

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. Because the TetR-expressing virus carries a blasticidin resistance gene, we did not generate an inducible CMV/TO lentiviral vector carrying the same drug resistance gene. We also generated an Entry vector with the TetR cDNA, pENTR/TetR (**559-1, Addgene #22265**) in case another drug selection is preferable. Please see section 5 "Transduction and Expression of the cDNA (page 46) for other considerations when making inducible expressions in T-Rex cell lines.

iv) pLenti GFP DEST

We sought to create a vector that would allow detection of transduced cells via GFP expression while simultaneously delivering a transgene of choice. To do this, we created a vector in which GFP expression is under the control of the CMV promoter and either a cDNA, shRNA or miRNA can be placed under the control of the EF-1α, H1, H1/TO, U6, CMV or CMV/TO promoters. GFP expression allows detection and/or isolation of transduced cells by either immunofluorescence or cell sorting. However, protein expression might be lower since the Gateway Destination cassette is inserted after the WPRE. Please refer to page ### for more details.

v) Retroviral Destination vectors

With the exception of the pEF-ENTR series, all the other Entry vectors are compatible with these retroviral vectors, so we can easily recombine inserts into either retroviral and lentiviral vectors. We replaced the CMV promoter from the original pQCXI plasmid with the CMV/TO promoter with the Gateway Destination cassette. We also inserted the zeocin and blasticidin resistance genes after the internal ribosome entry site (IRES) so we could use these drugs for selection. One possible drawback of these constructs is the control of the drug resistance gene by the CMV/TO promoter. In a T-REx cell line, both the cDNA and the drug resistance gene will be repressed, allowing selection of transduced cells only when the cDNA is

induced. However, these vectors can be used to transduce non-T-REx cells for constitutive expression. A construct in which only the transgene and not the drug resistance gene is inducible was designed by replacing the IRES with a murine PGK promoter to control the puromycin resistance gene from an independent and non-inducible promoter.

Our packaging 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 [16,17,18] 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 [19,20]. 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 [21]. 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 [22,23,24,25,26,27,28,29,30,31], 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.

I- Maps of the Entry Vectors

The Entry vectors are used to clone the cDNA and to later recombine it into the lentiviral backbone for either constitutive or inducible expression. The general Entry vectors pENTR1A, pENTR3C, pENTR4 (Invitrogen) have been used successfully with this system. Below are other vectors with either epitope tags (V5, FLAG), fusion proteins (eGFP, NTAP, Ubc9) for various applications and a constitutive physiological promoter, Elongation-Factor 1α (EF-1α). In the latter case, a promoter-less Destination vector has to be used (see below).

pENTR1A, no ccDB (w48-1, Addgene #17398)



Comment s for pENTR 1A no ccdB
2294 nucleotides

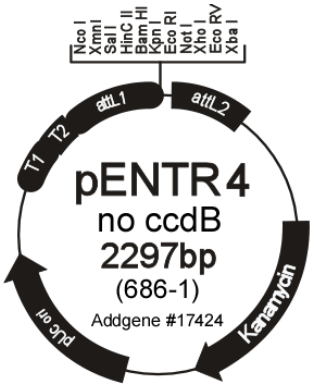
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
multiple cloning site (MCS): bases 457-522
attL2:bases 522-622
Kanamycin resistance gene: bases 745-1554
pUC origin: bases 1619-2291

Multiple cloning site of pENTR1A no ccdB:

```
Dra I          Xmn I      Sal I Hinc II  BamH I          Kpn I  EcoR I
TTT AAA GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC CGA ATT C
AAA TTT CCT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA G

     Not I          Xho I      EcoRV Xba I
GC GGC CGC CGC ACT CGA GAT ATC TAG ACC CAG CTT TCT TGT ACA AA
CG CCG GCG GCG TGA GCT CTA TAG ATC TGG GTC GAA AGA ACA TGT TT
```

pENTR4, no ccdB (686-1, Addgene #17424)



Comments for pENTR4 no ccdB
2297 nucleotides

rrnBT1 transcription termination sequence: bases 106-149
rrnBT2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
multiple cloning site (MCS): bases 461-525
attL2: bases 526-626
Kanamycin resistance gene: bases 748-1557
pUC origin: bases 1666-2294

Multiple cloning site of pENTR4 noccdB:

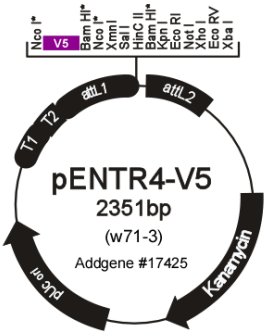
```
Nco I          Xmn I      Sal I  HinC II  Bam HI     Kpn I      EcoR I     Not I
ACC ATG GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC CGA ATT CGC GGC CGC
TGG TAC CCT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA GCG CCG GCG

Xho I      Eco RV Xba I
ACT CGA GAT ATC TAG ACC AGC TTT
TGA GCT CTA TAG ATC TGG TCG AAA
```

The pENTR1A no ccdB and pENTR4 no ccdB vectors can be used to clone untagged cDNAs or cDNAs already containing a tag. The ccdB gene was removed from the original vectors so the full multiple cloning site could be used.
*** Please note that the pENTR4 no ccdB vector contains an *Nco* I site (CCATGG) in its multiple cloning site which has an ATG that could be used as an initiation codon. If the cDNA is cloned after the *Nco* I site, it should be in frame with the ATG. I normally avoid using the ENTR4 vector unless I am using the Nco I site.

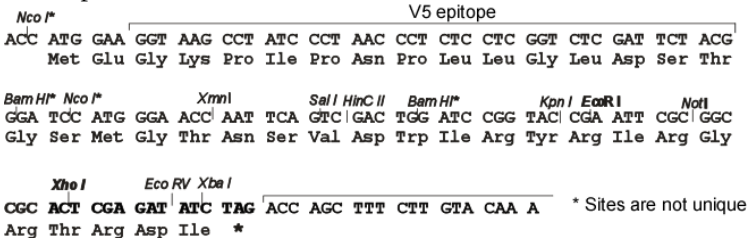
The pENTR1A noccDB and pENTR4 noccDB vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (See Table 1 for details).

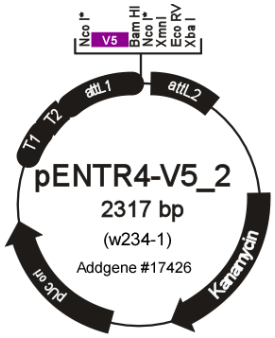
pENTR4-V5, V5-2 vectors (Addgene #17425, 17426)



rrnBT1 transcription termination sequence: bases 106-149
rrnBT2 transcription termination sequence: bases 281-308
att L1: bases 358-457 (complementary strand)
V5 epitope: bases 469-510
Multiple cloning site (MCS): bases 511-579
attL2: bases 580-680
Kanamycin resistance gene: bases 802-1611
pUC origin: bases 1677-2348

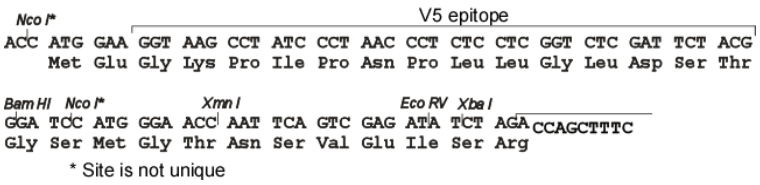
Multiple cloning site of pENTR4-V5:





rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
att L1: bases 358-457 (complementary strand)
V5 epitope: bases 469-510
Multiple cloning site (MCS): bases 511-545
attL2: bases 546-646
Kanamycin resistance gene: bases 768-1577
pUC origin: bases 1642-2314

Multiple Cloning Site of pENTR4-V5_2:



These two vectors are essentially the same except for a slight variation in their MCS. Both allow expression of the candidate cDNA fused to an N-terminal V5 epitope. The pENTR4-V5 was digested with Sal I/Xho I and recircularized to generate pENTR4-V5_2. It removes one of the two Bam HI site for easier subcloning through the Bam HI site which is found in several other cloning vectors. We normally use the Invitrogen's mouse anti-V5 antibody (cat#R960-25) for Western blots and Immunofluorescence and Bethyl Laboratories rabbit anti-V5 antibody (cat.#A190-120A) for Western blots. Figure 6 shows HeLa V5-Luc cell lines made with different

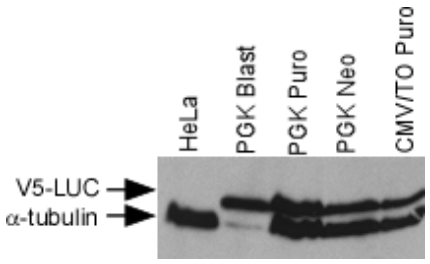
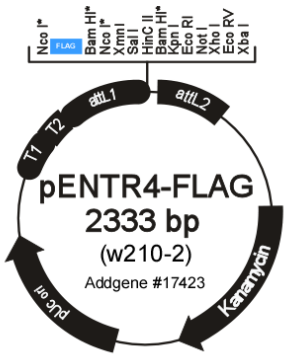


Figure 6. Generation of viruses expressing V5-luciferase using the pENTR4-V5 vector. The Firefly luciferase cDNA was inserted into the pENTR4-V5 plasmid and recombined with either the pLenti PGK Blast DEST (w524-1), pLenti PGK Puro DEST (w529-2), pLenti PGK Neo DEST (w531-1) or pLenti CMV/TO Puro (670-1) plasmids. Viral particles were used to transduce HeLa cells and transduced cells were selected with the appropriate antibiotic. Cellular extracts were made for Western blots and probed for the V5 fusion. α-tubulin is used as a loading control.

lentiviral vectors. The V5 epitope has been used successfully for Western blots, immunofluorescence, immunoprecipitations and chromatin immunoprecipitations (ChIP).

The pENTR4-V5 vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

pENTR4-FLAG vector (w210-2, Addgene #17423):



Comment s for pENTR 4-FLAG
2333 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
att L1: bases 358-457 (complementary strand)
FLAG epitope: bases 463-489
Multiple Cloning Site: bases 493-561
attL2:bases 562-662
Kanamycin resistance gene: bases 784-1593
pUC origin: bases 1701-2330

Multiple Cloning Site of pENTR4-FLAG:

```
Nco I*                FLAG epitope                          Bam HI* Nco I*
ACC ATG GAT TAC AAG GAC GAT GAC GAC AAA CTC GGA TCC ATG
    Met Asp Tyr Lys Asp Asp Asp Asp Lys Leu Gly Ser Met

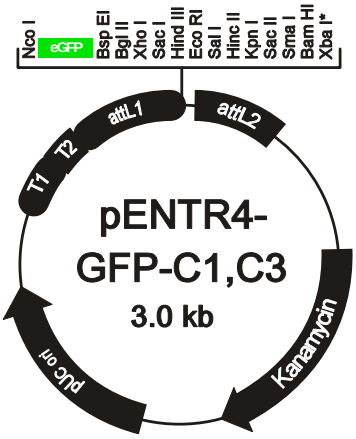
  Xmn I          Sal I HinC II  Bam HI*        Kpn I EcoR I      Not I
GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC CGA ATT CGC GGC
Gly Thr Asn Ser Val Asp Trp Ile Arg Tyr Arg Ile Arg Gly

    Xho I      Eco RV Xba I
CGC ACT CGA GAT ATC TAG ACCAGCTTT
Arg Thr Arg Asp Ile  *
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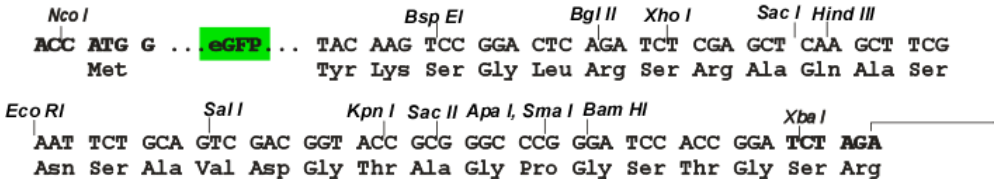
This plasmid allow expression of the target protein with an N-terminal FLAG epitope. We normally use the anti-FLAG M2 antibody from Sigma for Western blots. The FLAG epitope has been successfully used for Western Blots, immunofluorescence, immunoprecipitations and chromatin immunorepcipitations (ChIP).

The pENTR4-FLAG vector is compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

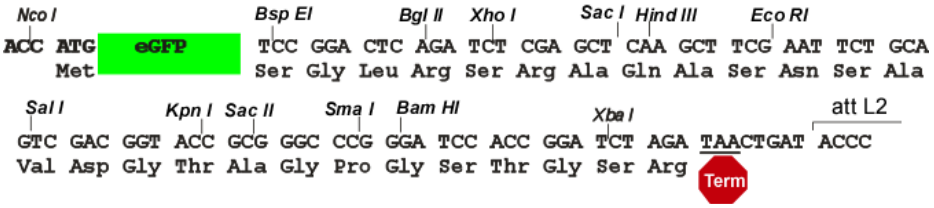
pENTR4-eGFP-C1, C1X, C3 Vectors (w392-1, w393-1, Addgene #17396, 22450, 17397):



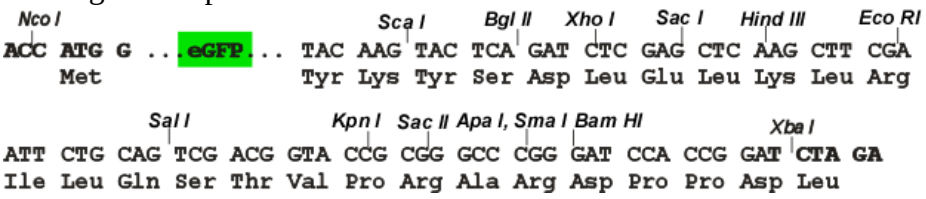
Multiple Cloning Site of pENTR4-eGFP-C1:



Multiple Cloning Site of pENTR4-eGFP-C1X:

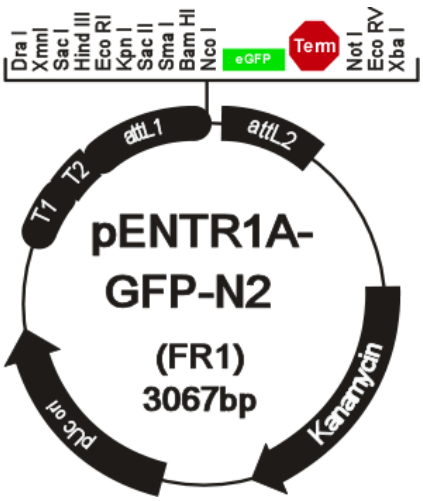


Multiple Cloning Site of pENTR4-eGFP-C3:



The eGFP-MCS portion of the peGFP-C1 or peGFP-C3 (Clontech) plasmids were cloned into the Nco I/Xho I sites of pENTR4. The pENTR4-eGFP-C1X plasmid was made by the Nathan Lawson laboratory (University of Massachusetts Medical School) under the name pENTREGFP2. It includes a termination codon unlike the other two Entry vectors. These plasmids are useful to fuse the protein of interest at the C-terminus of GFP. The pENTR-eGFP-C2 vector is not made presently but the same cloning strategy could be used to generate this frame variant.

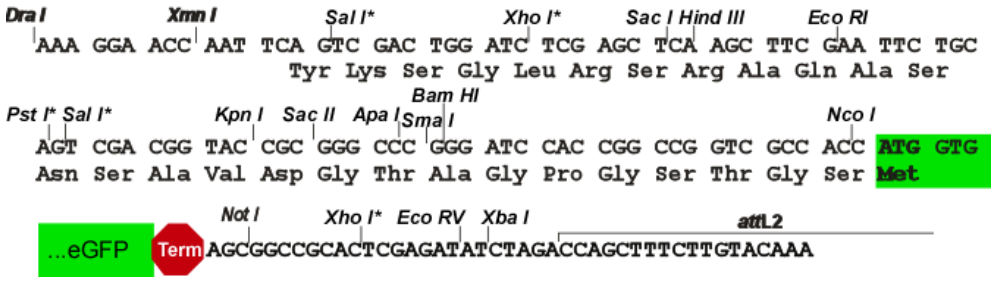
pENTR1-GFP-N2 (FR1, Addgene #19364)



Comments for pENTR1A-GFP-N2
3067 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
enhanced green fluorescent protein (eGFP): bases 552-1271
attL2:bases 1296-1396
Kanamycin resistance gene: bases 1518-2327
pUC origin: bases 2392-3064

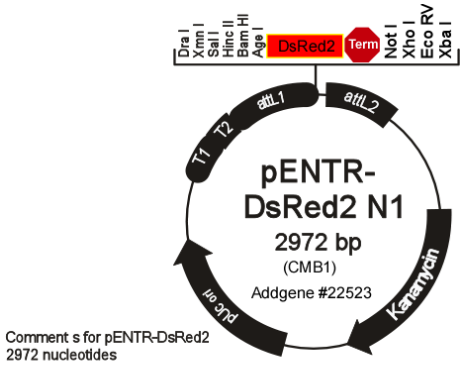
Multiple cloning site of pENTR1-GFP-N2:



This vector can be used to express the protein of interest fused with GFP at its C-terminus and also as a GFP expression vector when it is recombined with a suitable Destination vector. This vector was made by Francis Rodier (Université de Montréal).

The pENTR-GFP vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

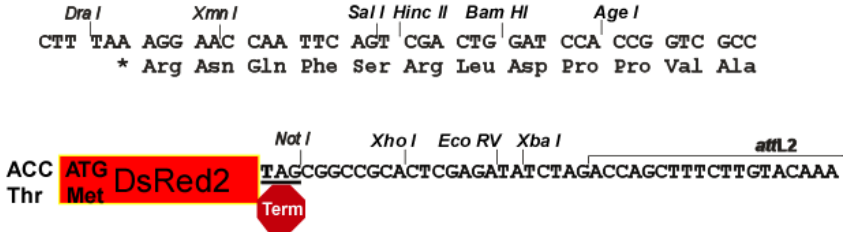
pENTR-DsRed2-N1 and DsRedEx2-C1 (CMB1, Addgene #22523, 22451)

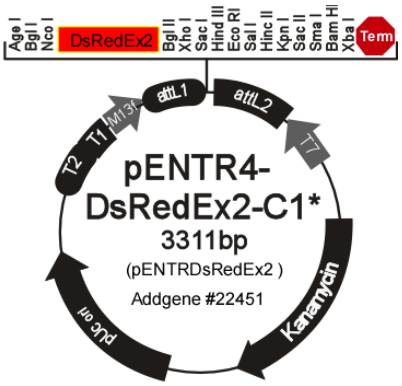


Comment s for pENTR-DsRed2
2972 nucleotides

rrnBT1 transcription termination sequence: bases 106-149
rrnBT2 transcription termination sequence: bases 281-308
attL1:bases 358-457
Multiple cloning site (MCS) bases 457-493
DsRed2 fluorescent cDNA: bases 501-1178
attL2: bases 1201-1301
Kanamycin resistance gene: bases 1423-2232
pUC origin: bases 2297-2969

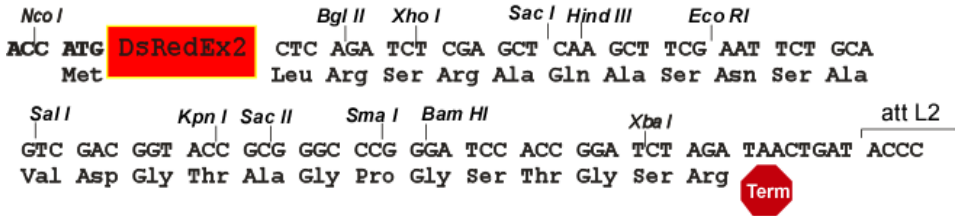
Multiple Cloning Site of pENTR-DsRed2 N1 (CMB1):





Comment s for pENTR4-DsRedEx2-C1
3311 nucleotides
rrnB T2 transcription termination sequence: bases 295-268
rrnBT1 transcription termination sequence: bases 470-427
M13forw (-20): 537-552
attL1: bases 568-668 (complementary strand)
DsRed express cDNA: bases 686-1360
Multiple cloning site (MCS): bases 1361-1429
Termination codon: bases 1430-1432
att L2: bases 1438-1538
T7 promoter: 1573-1554
Kanamycin resistance gene: bases 1707-2516
pUC origin: bases 2638-3311

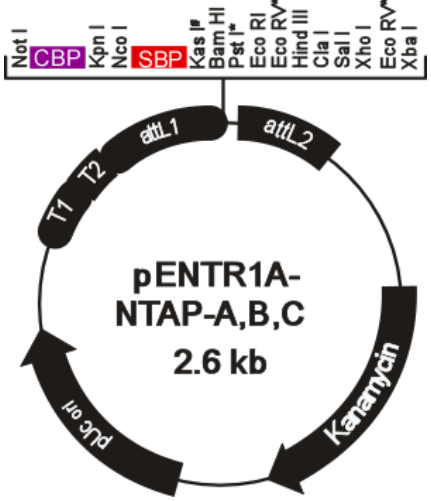
Multiple Cloning Site of pENTR4-DsRedEx2-C1X:



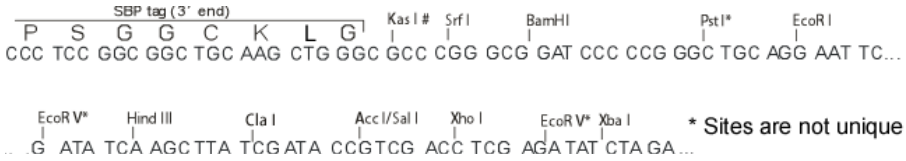
Similar to the pENTR-GFP N2 fusion vectors, this vector can either be used as a C-terminal fusion with DsRed2 or to make cells red fluorescent when recombined with either the pLenti CMV, CMV/TO or PGK series. **DsRed-Express** is a rapidly maturing variant of *Discosoma sp.* red fluorescent protein (DsRed) that contains nine amino acid substitutions which enhance its solubility, reduce its green emission, and accelerate its maturation. Although DsRed-Express most likely forms the same tetrameric structure as wild-type DsRed, DsRed-Express displays a reduced tendency to aggregate. **DsRed-Express2** has higher solubility, and was designed to be better suited for cell and stem cell applications (taken fron Clontech's web site). Link here. The pENTR DsRed2-N1 vector was made by Christian Beauséjour (Université de Montréal) and the pENTR4-DsRedEx2-C1 by the Nathan Lawson laboratory (University of Massachusetts Medical School).

The pENTR-DsRed vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

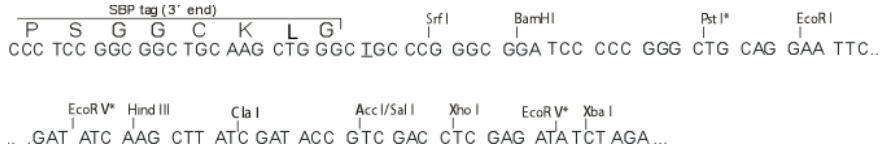
pENTR1A-NTAP-A,B,C vectors (w322-1, w323-1, w324-1):



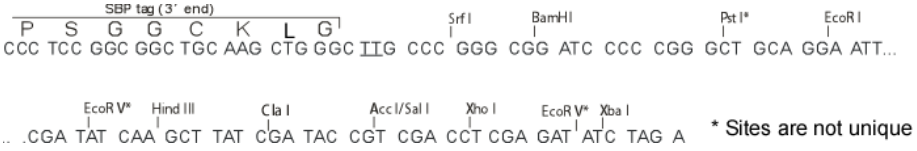
Multiple cloning site of pENTR1A-NTAP-A (w322-1, Addgene #17402):



Multiple Cloning Site of pENTR1A-NTAP-B (w323-1, Addgene #17403):



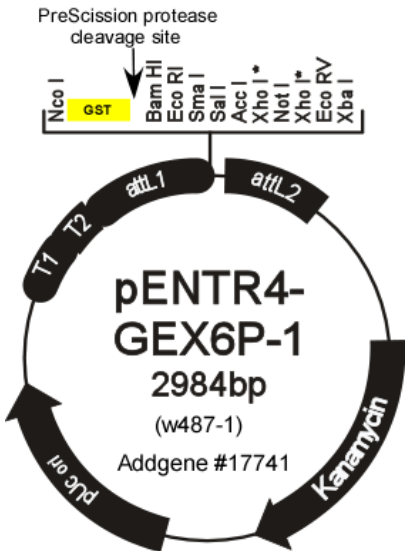
Multiple Cloning Site of pENTR1A-NTAP-C (w324-1, Addgene#17404):



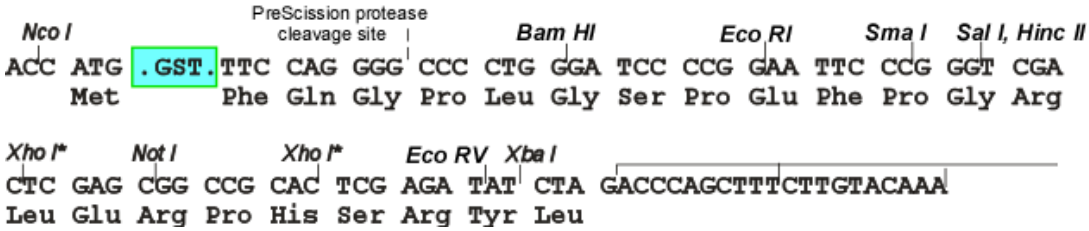
These plasmids are derived from the Interplay Mammalian TAP System from Stratagene (link here) (pNTAP-A,B,C) in order to isolate protein complexes interacting with the protein of interest. The method is based on expression of a protein fused to two affinity tags: a streptavidin binding peptide (SBP) and a calmodulin binding peptide (CBP). Tandem affinity purification yields tagged protein and interacting proteins using gentle washing and small molecule elution conditions. Protocols to purify protein complexes can be found on Stratagene's web site our in our article describing the use of these vectors [5].

The pENTR-NTAP vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

pENTR4-GEX-6P-1 vector (w487-1, Addgene #17741):



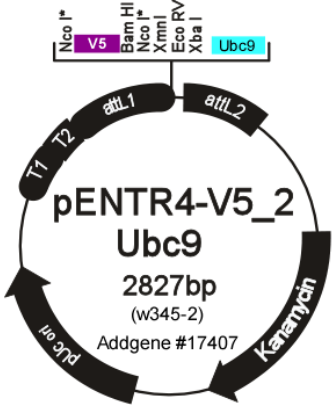
Multiple Cloning Site of pENTR4-GEX-6P-1 (w487-1, Addgene #17741):



This vector is based on the pGEX-6P-1 vector from GE Healthcare. It fuses the glutathione-S-transferase (GST) to the protein for purification on glutathione resin. The GST tag can be cleaved by incubating the fusion protein with the PreScission protease. Please refer to the GST Gene Fusion System from GE Healthcare for more details.

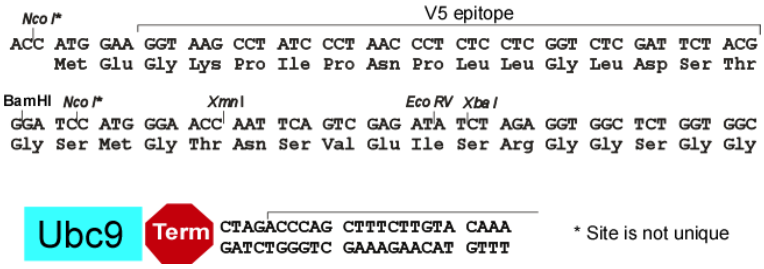
The pENTR4-GEX6P-1 vector is compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

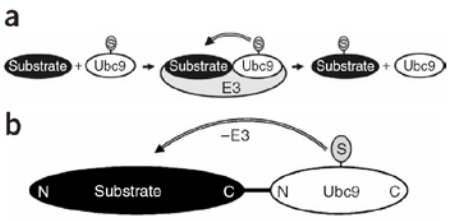
pENTR4-V5_2-Ubc9 vector (w345-2, Addgene #17407):



rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
V5 epitope: 463-510
Multiple cloning site (MCS): bases 511-546
Ubc9 cDNA: 563-1039
attL2:bases 1058-1158
Kanamycin resistance gene: bases 1280-2089
pUC origin: bases 2153-2824

Multiple Cloning Sites of pENTR4-V5_2 Ubc9:





The (Ubc9) fusion-directed SUMOylation (UFDS) system is used to detect Sumoylation of the target protein by fusing it directly to the E2 SUMO ligase Ubc9 ([10]. This allows detection of Sumoylated proteins that can be normally found at very low levels and bypasses the need for the E3 SUMO ligase (b, above taken from [10]). Detailed protocols on the use of the UFDS have been published [32].

The pENTR4-V5-Ubc9 vector is compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

FKBP12DD (Destabilization Domain) Vectors:

The Wandless laboratory (http://wandless.stanford.edu/) engineered mutants of the human FKBP12 protein that are rapidly degraded when expressed in mammalian cells [9], and this instability is conferred to other proteins when they are fused to these destabilizing domains. Addition of a synthetic ligand (Shield-1) that binds to the destabilizing domains shields them from degradation, allowing fused proteins to perform their cellular functions. Genetic fusion of the destabilizing domain to a gene of interest ensures specificity, and the Shield-1 control confers speed, reversibility, and dose-dependence to this method. Adding back Shield-1 will allow re-expression of the fusion protein. The mutant that conferred the most rapid degradation was the L106P mutant. (Figure 7A taken from [33]. The system has also been commercialized by Clontech under the name ProteoTuner system. Please refer to Clontech's web site for more details (here). The Shield-1 ligand can be bought from either Clontech or CheminPharma. As an example, Asf1a was fused to the FKBP-DD domain and shown to be degraded upon removal of Shield-1 (Figure7B). We also used the FKBP-Asf1a fusion to show that we could induce either expression or degradation of the FKBP-Asf1a protein in cells arrested in mitosis [5]. Therefore, this system allows very rapid (between 1-5 hours) and reversible expression or degradation of the protein of interest, even in mitotically arrested cells

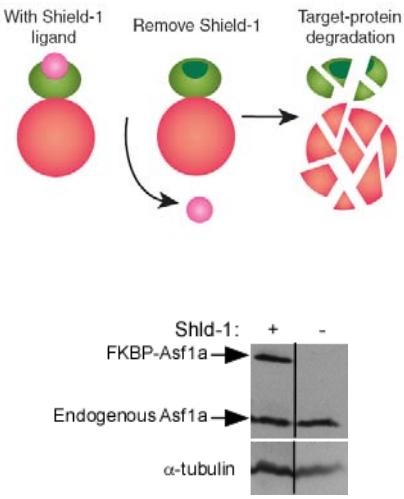
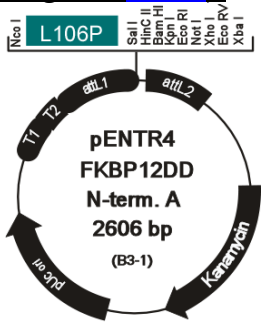


Figure 7. The FKBP-DD (Proteotuner) system. (A) Overview of the system. The protein of interest (pink) is shielded from degradation in the presence of the Shield-1 ligand. Upon removal of Shield-1, the protein is rapidly degraded. **(B)** Fusion of the histone chaperone Asf1a to FFKP-DD. The FKBP-Asf1a fusion protein can be detected in the presence of Shield-1 but not in its absence. Endogenous levels of Asf1a don't change during expression or depletion of FKBP-Asf1a. Alpha-tubulin is used as a loading control. Other results of the FKBP-Asf1a fusion are shown in the paper describing these vectors [5].

Maps of pENTR4-FKBP12DD vectors

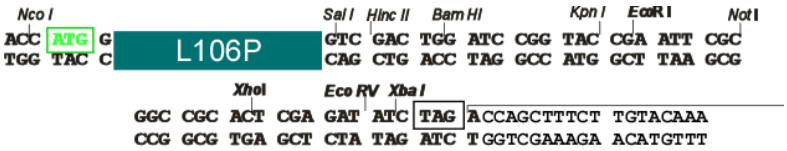
pENTR4-FKBP12DD N-term A (B3-1, Addgene #17414):



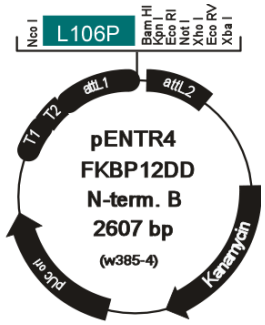
Comments for pENTR4 FKBP12DD N-term A 2606 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
FKBP12DD (L106P) domain: 463-786
Multiple cloning site (MCS): bases 787-834
attL2:bases 835-935
Kanamycin resistance gene: bases 1057-1866
pUC origin: bases 1932-2603

MCS (B3-1):



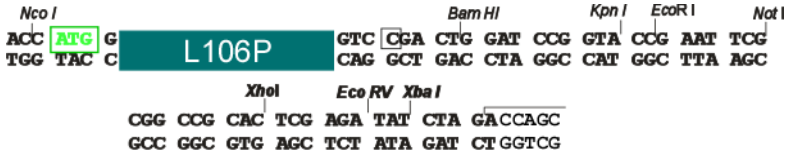
pENTR4-FKBP12DD N-term B (w385-4, Addgene #17415):



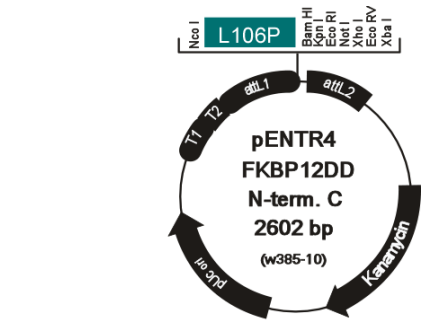
Comments for pENTR4 FKBP12DD N-term B 2607 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
FKBP12DD (L106P) domain: 463-786
Multiple cloning site (MCS): bases 787-835
attL2:bases 836-936
Kanamycin resistance gene: bases 1058-1867
pUC origin: bases 1933-2604

MCS (w385-4):



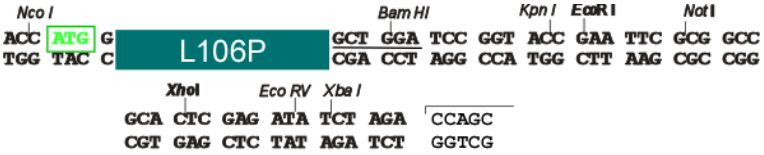
pENTR4-FKBP12DD N-term C (w385-10, Addgene #17416):



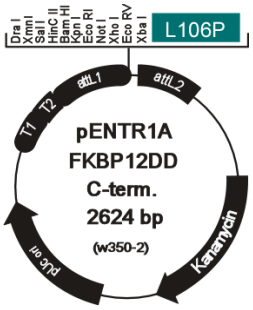
Comments for pENTR4 FKBP12DD N-term C 2602 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
FKBP12DD (L106P) domain: 463-786
Multiple cloning site (MCS): bases 786-830
attL2:bases 831-931
Kanamycin resistance gene: bases 1053-1862
pUC origin: bases 1928-2599

MCS (w385-10):



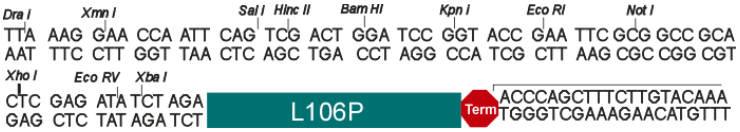
pENTR4-FKBP12DD C-term (w350-2, Addgene #17413):



Comments for pENTR 1A FKBP12DD C-term 2624 nucleotides

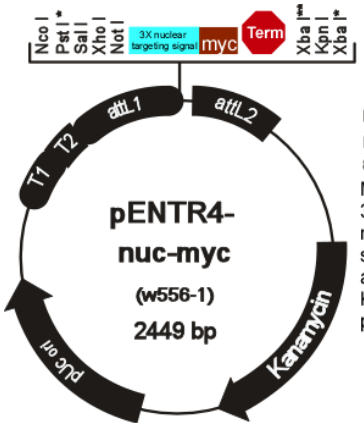
rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
Multiple cloning site (MCS): bases 457-523
FKBP12DD (L106P) domain: 524-847
attL2:bases 853-953
Kanamycin resistance gene: bases 1075-1884
pUC origin: bases 1949-2621

MCS (w350-2):



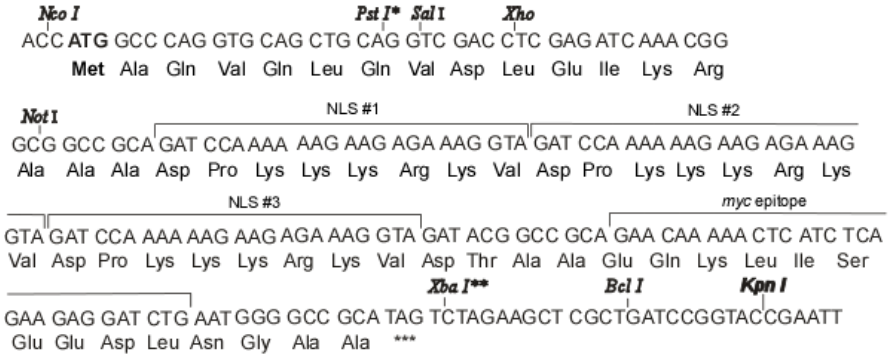
The pENTR-FKBP12 DD vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

pENTR4-nuc-myc (w556-1, Addgene #20026):



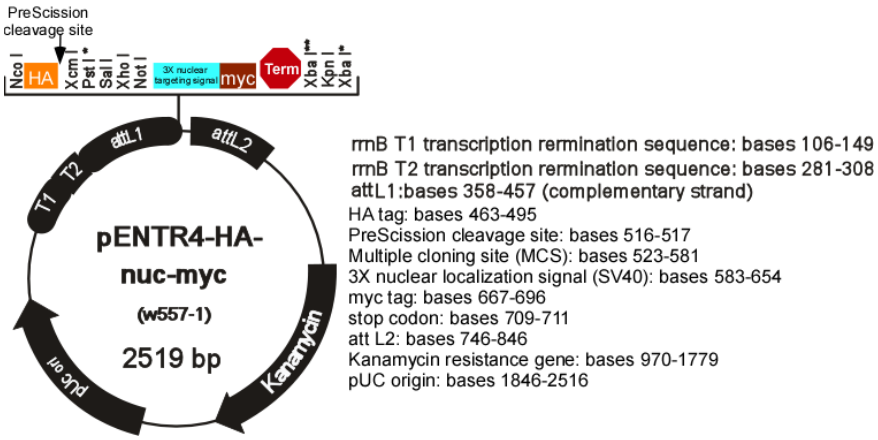
rrnB T1 transcription rermination sequence: bases 106-149
rrnB T2 transcription rermination sequence: bases 281-308
attL1:bases 358-457 (complementary strand)
Multiple cloning site (MCS): bases 461-512
3X nuclear localization signal (SV40): bases 514-585
myc tag: bases 598-627
stop codon: bases 640-642
att L2: bases 679-779
Kanamycin resistance gene: bases 900-1709
pUC origin: bases 1774-2446

Multiple cloning site for pENTR4-nuc-myc (w556-1):

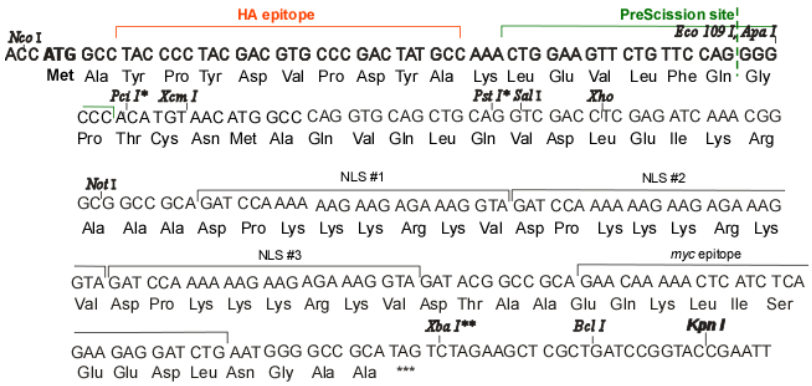


*Site is not unique
** Site is methylated in original vector (pCMV/myc/nuc)

pENTR4-HA-nuc-myc (w557-1, Addgene #20025):



Multiple cloning site pENTR4-HA-nuc-myc (w557-1):

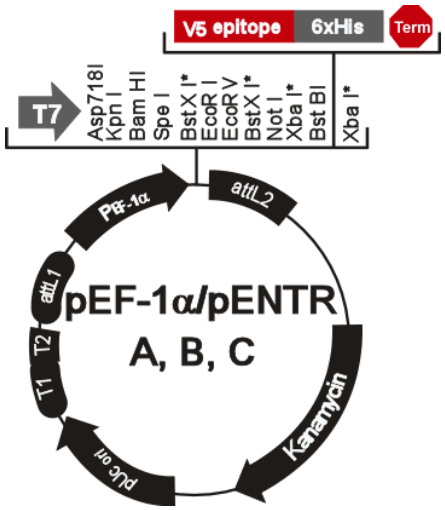


*Site is not unique
** Site is methylated in original vector (pCMV/myc/nuc)

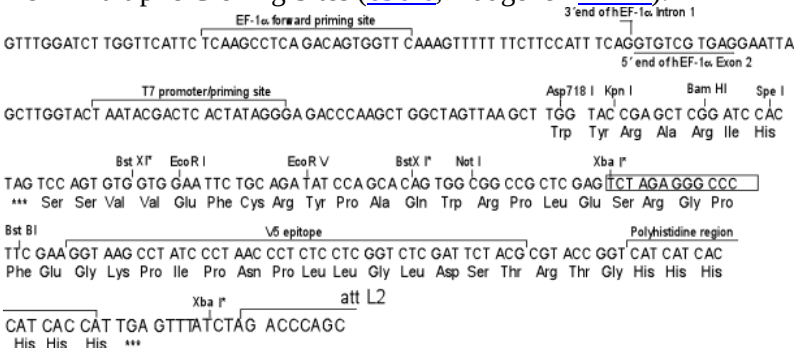
These two vectors allow targeting of the protein to the nucleus through a C-terminal fusion with three nuclear localization signals from the SV40 virus. It also contains a C-terminal myc fusion protein for detection or purification of the protein. The pENTR4-HA-nuc-myc also has an N-terminal HA tag also for detection or purification. These vectors are derived from the pCMV myc nuc vector from Invitrogen (pShooter vectors). Please refer to Invitrogen's web site for more details (here).

The pENTR4 nuc-myc vectors are compatible with the pLenti CMV, CMV/TO, PGK series, and pQCX CMV/TO series of Destination vectors (see Table 1 for details).

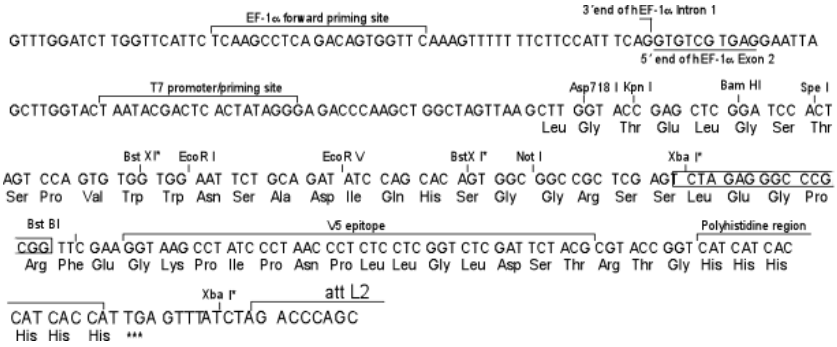
pEF-1α/pENTR Vectors:



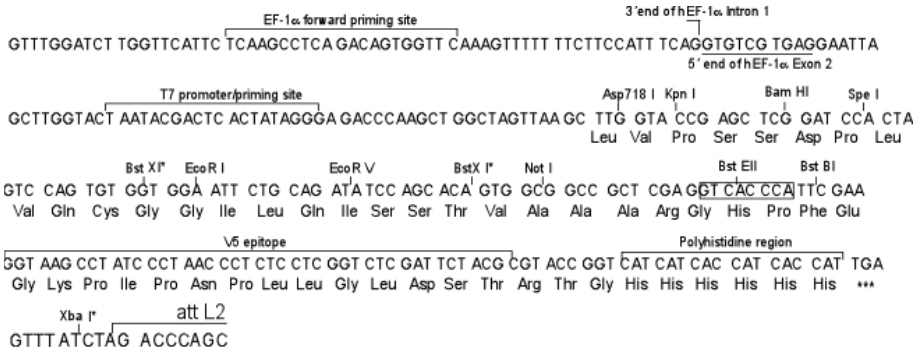
pEF-ENTR Frame A Multiplie Cloning Sites (696-6, Addgene #17427):



pEF-ENTR Frame B Multiplie Cloning Sites (708-1, Addgene #17428):



pEF-ENTR Frame C Multiplie Cloning Sites (709-1, Addgene #17429):



The human elongation factor 1α (EF-1α) allows high level constitutive expression in a wide range of mammalian cells. Furthermore, the expression of the promoter is not sensitive to promoter methylation which can occur for the CMV promoter. To express the cDNA from these plasmids, a promoter-less Destination vector such as the pLenti X1 series has to be used. Refer to Table 1 (page 9 and 29) for more details on Entry to Destination vector compatibilities.

The pEF-ENTR vectors are only compatible with the pLenti X1 series of Destination vectors (see Table 1 for details).

II-Maps of the Lentiviral Destination Vectors

These Destination vectors contain the central polypurine tract (cPPT) and the Woodchuck Post-Transcriptional Response Element (WPRE) for higher viral titers and transduction efficiencies [1,2,3,4]. Using the Gateway technology, each Entry vector can be recombined into multiple retroviral and lentiviral Destination vectors. However, not all combinations of Entry and Destination vectors are meant to be recombined. Because the pEF-ENTR series vectors contain a promoter and not the other vectors, different Destination vectors will be compatible. The compatibilities of each Entry vector for various Destination vectors for expression studies are summarized in Table 1 below as well as represented schematically in Figure 8 for lentiviruses, and Figure 9 for retroviruses.

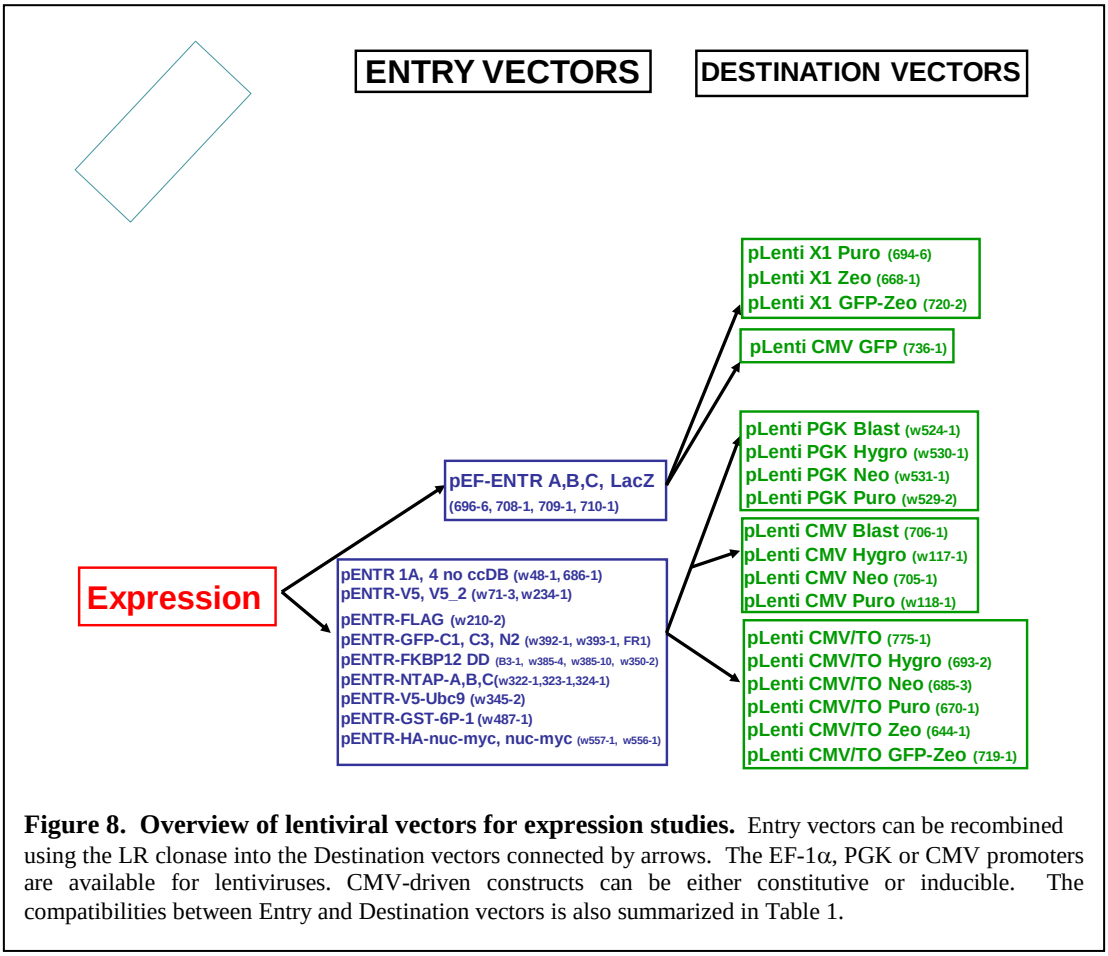


Figure 8. Overview of lentiviral vectors for expression studies. Entry vectors can be recombined using the LR clonase into the Destination vectors connected by arrows. The EF-1α, PGK or CMV promoters are available for lentiviruses. CMV-driven constructs can be either constitutive or inducible. The compatibilities between Entry and Destination vectors is also summarized in Table 1.

Table 1: Compatibilities of the Entry to Destination vectors for Expression Studies

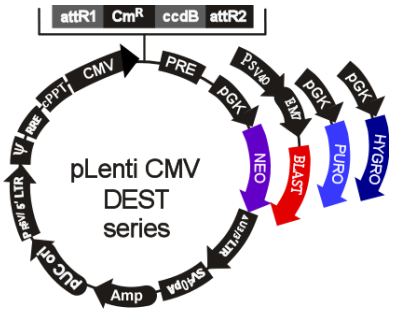
Entry vector type	Compatible Destination vectors	Incompatible Destination vectors
pEF-ENTR Series	pLenti X1 Series pLenti CMV GFP	pLenti X2 Series[b] pLenti PGK Series[a] pLenti CMV Series[a] pLenti CMV/TO Series[a] pQCXI CMV/TO Series[a] pQCXI RNAi X2 Series[b]
pENTR-Fusion Series	pLenti PGK Series pLenti CMV Series pLenti CMV/TO Series pQCXI CMV/TO Series	pLenti X1 Series[c] pLenti X2 Series[b,c] pLenti CMV GFP[c] pQCXI RNAi X2 Series[b,c]
[a]Probable promoter interference. [b]Insert would be too big for the 3'LTR. [c]No promoter to drive expression of the cDNA.		

The cDNAs can easily be swapped between the inducible and constitutive backbones as well as for the various drug selections. However, not all combinations of Entry to Destination vector are compatible. The pLenti CMV and pLenti PGK vectors allow constitutive expression in any cell line, whereas the pLenti CMV/TO vectors allow inducible expression in a T-REx cell line. Please note that expression of the pLenti CMV/TO vectors in a cell line that is not T-Rex will confer constitutive expression of the cDNA. This is summarized in Table 2 below:

Table 2: Capacity of expression vectors for inducible expression in a T-REx cell line.

Vector	Inducible expression in a T-REx cell line
pLenti CMV Series	NO
pLenti CMV/TO Series	YES
pLenti PGK Series	NO
pQCXI CMV/TO Series	YES*
pQCXP	YES
*Both the cDNA and the drug resistance gene will be under the control of the CMV/TO promoter, except for pQCXP.	

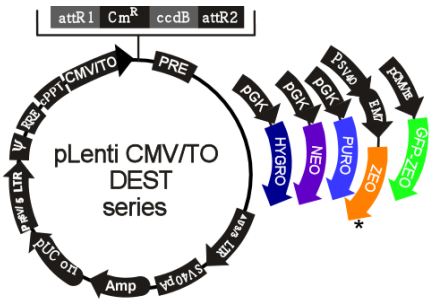
pLenti CMV Vectors:



Blast: 706-1, Addgene #17451
Hygro: w117-1, Addgene #17454
Neo: 705-1, Addgene #17392
Puro: w118-1, Addgene #17452

Example of pLenti CMV Hygro vector: Figure 7.

pLenti CMV/TO Vectors:

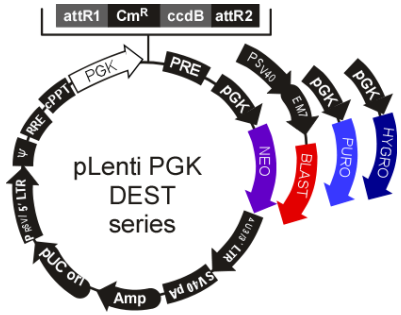


Hygro:693-2, Addgene #17291
Neo: 685-3, Addgene #17292
Puro: 670-1, Addgene #17293
Zeo*:644-1, Addgene #17294
CMVIE-GFP-Zeo: 719-1, Addgene #17431

*These versions do not have the ampicillin gene. Use zeocin in low salt LB when growing in *E.coli*.

Example of pLenti CMV/TO Puro vector: Figure 6.

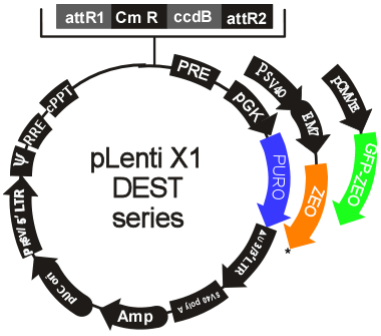
pLenti PGK Vectors :



Blast: w524-1, Addgene #19065
Hygro: w530-1, Addgene #19066
Neo: w531-1, Addgene #19067
Puro: w529-2, Addgene #19068

Examples of pLenti PGK Blast, Neo, Puro vectors: Figure 6.

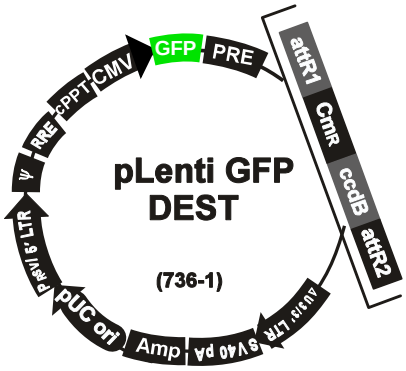
pLenti X1 Vectors:



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

Since these vectors do not have a promoter in front of the Gateway cassette, they can only be used with Entry vectors that contain a promoter like the pEF-ENTR series. It is also possible to clone a promoter with a cDNA into the pENTR1A noccDB vector and recombine it with the pLenti X1 series. These vectors can also be used with the vectors for RNA interference (see the Manual for RNAi studies for details, at http://ericcampeau.com/manuals.html).

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



As mentioned in the background section, this vector can be useful to monitor transduced cells since they will express GFP. Also, the introduction of the WPRE element (shown as PRE in the above map) increases the levels of expressed protein when placed at the 3' end of the cDNA. In this vector, the GFP should benefit from the WPRE. However, the Gateway Destination cassette is inserted after the WPRE so the protein of interest might not be expressed at the same levels as in the pLenti CMV, CMV/TO or PGK series. We did not directly compare the expression of a protein in the pLenti CMV, CMV/TO or PGK series vs the pLenti GFP DEST at the time of writing of this manual. We will include this information in an updated version of this manual when it will be available. Nonetheless, we expect this vector should have similar depletion efficiencies to the pLenti X1 series if an H1 promoter-shRNA cassette is inserted after the WPRE because this element has no reported beneficiary effect on RNA polymerase III transcription. Furthermore, the pLK0.1 vector used by the RNAi consortium works very well and does not have any WPRE. Although we obtained comparable viral titers between various lentiviral vectors (pLK0.1, pFSIPPW, pGIPZ, [5]), we did not directly compare depletion efficiencies amongst these vectors.

III-Maps of the Retroviral Destination Vectors

As mentioned previously, Entry vectors can also be recombined with different retroviral Destination vectors. However, as for the lentiviral Destination vectors, not all combinations will yield a functional vector. The compatibilities of the various Entry vectors to the various retroviral Destination vectors are summarized in Table 1 (page 30) and in Figure 9 below:

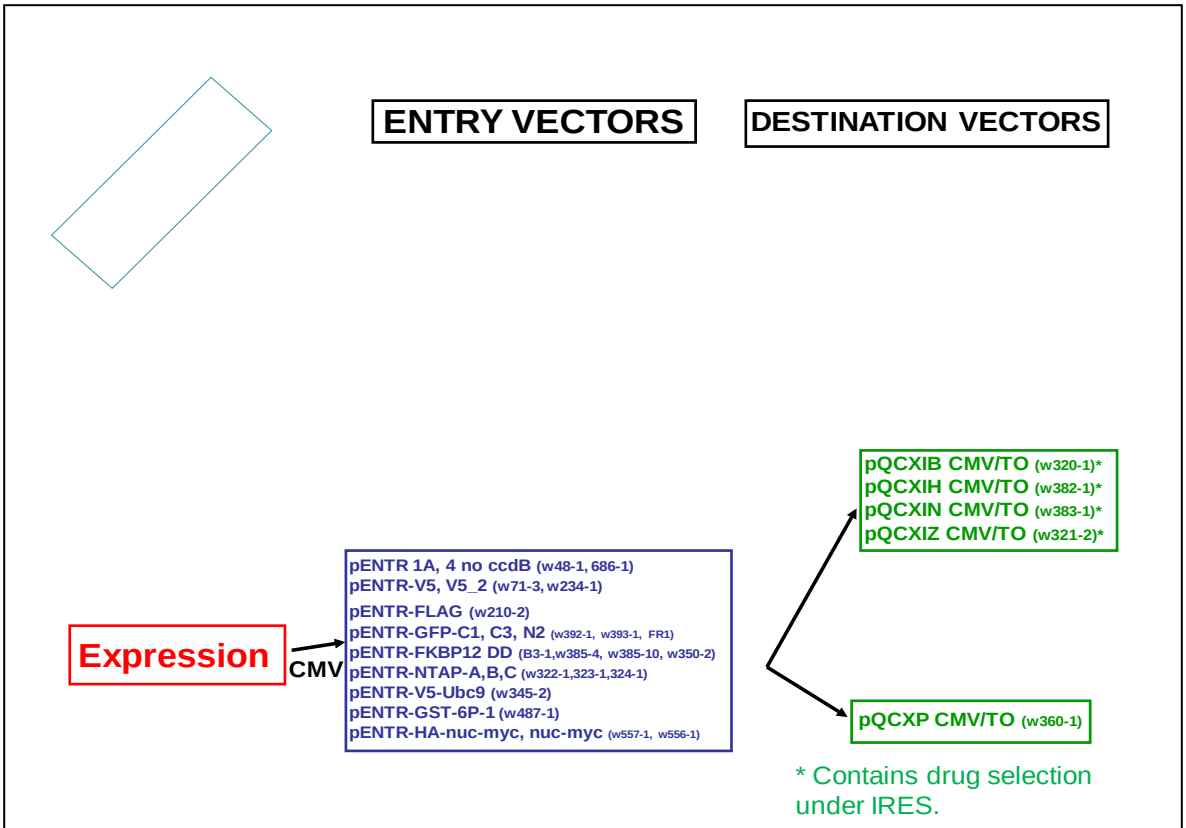
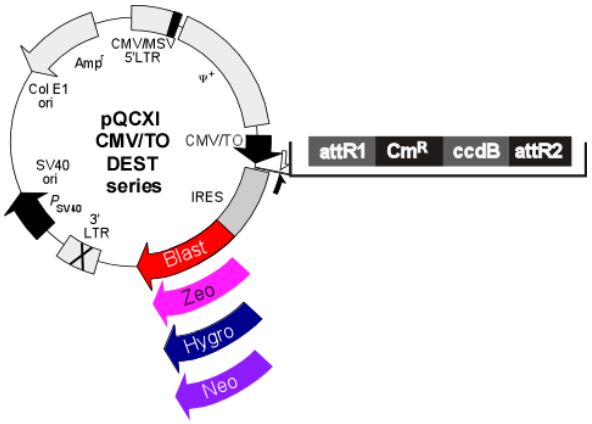


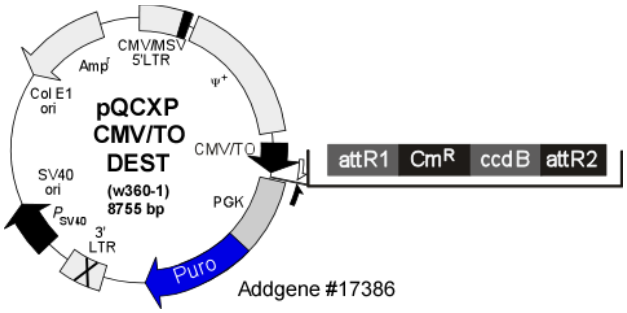
Figure 9. Overview of retroviral vectors for expression studies. Entry vectors can be recombined using the LR clonase into the Destination vectors connected by arrows. For the retroviral vectors, no constitutive expression of cDNAs can be attained in T-REx cell lines using the pQCXI series vectors because they all contain the inducible CMV/TO promoter. Similarly, the drug resistance gene will be repressed in a T-REx cell line since it is after an IRES element under the control of the CMV/TO promoter. However, the pQCXP CMV/TO can be used for inducible expression of cDNAs under constitutive puromycin selection in T-REx cell lines. See Table 2 for details.

pQCXI CMV/TO series:



Blast: w320-1, Addgene #17400
Hygro: w382-1, Addgene #17394
Neo: w383-1, Addgene #17395
Zeo: w321-2, Addgene #17401

pQCXP CMV/TO DEST (w360-1, Addgene #17386):



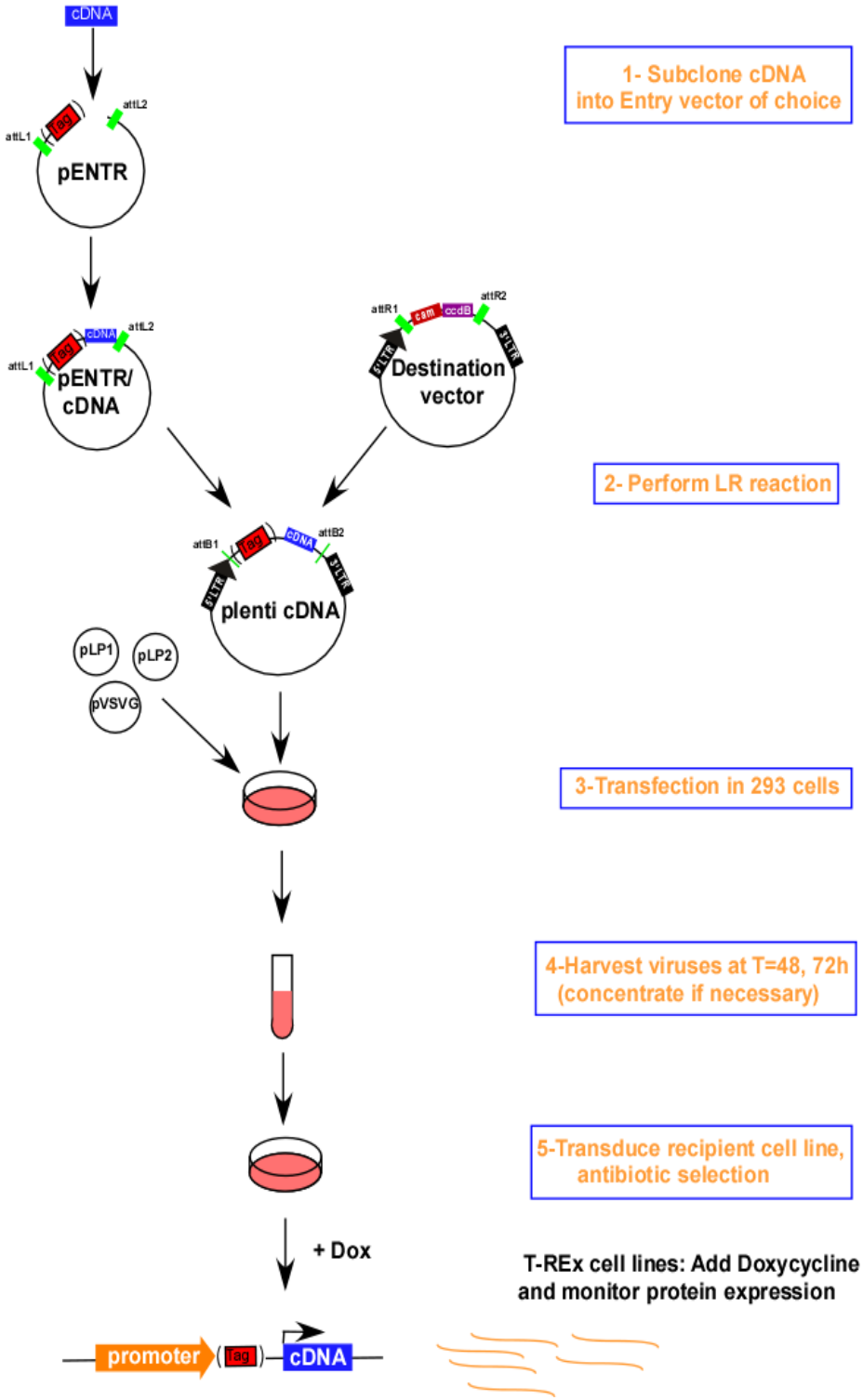
Addgene #17386

These vectors can be used as an alternative to the lentiviral vectors to express the protein of interest. In our hands, the titers obtained when generating viruses from these vectors are similar to the lentiviral vectors i.e ~10^5 cfu/ml with cDNAs <1.5kb. Larger cDNAs will show reduced viral titers, with an upper limit of ~5 kb for successful packaging.

IV-Compatibilities of these vectors to other Gateway vectors

The insert from the Entry vector clone is recombined via an attL-attR (LR) reaction [34] 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 [6], 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 [35,36,37]. Other Gateway Entry and Destination vectors can be used with this system. To be compatible with our system, other Entry vectors need to have the attL1 and attL2 recombination sites and any antibiotic selection in *E.coli* except ampicillin. For other Destination vectors, they need the attR1 and attR2 recombination sequences and any antibiotics resistance except kanamycin.

Experimental Outline



1- Subclone cDNA into Entry vector of choice

2- Perform LR reaction

3-Transfection in 293 cells

4-Harvest viruses at T=48, 72h (concentrate if necessary)

5-Transduce recipient cell line, antibiotic selection

T-REx cell lines: Add Doxycycline and monitor protein expression

.

V-Methods

1- Cloning of the cDNA into the Entry vector.

1. Propagate the Entry vectors in a general cloning strain such as *E.coli* TOP10F'. Propagate the Destination vectors in DB3.1 or ccDB survival strain (These strains can be obtained from Invitrogen).

2. Take 10 ul of the miniprep, 7 ul of H2O, 2 ul of 10X restriction buffer and 1 ul (5-20 units) of the restriction endonuclease.

3. If the plasmid has to be digested with two enzymes, add 1 ul of each restriction endonuclease. Try to choose a buffer that will be compatible with both enzymes. You can try buffers from different companies (even though the enzyme is not from that company, it generally works). 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, NEB #2 can easily be changed to NEB #3 by adding 1 ul of 1M NaCl. If not, use a Qiaquick column to exchange buffers.

4. Incubate for ~ 1 hour to overnight.

5. Treat the Entry vector with calf intestine alkaline phosphatase (CIAP). I usually add 2 ul of CIAP (either NEB's or Pharmacia's) and incubate it at 37^{o}C for 15 min. This will work in NEB #2,3,4 EcoRI and BamHI buffers but not NEB#1.

6. Add 2 ul of 6X agarose loading dye to the digested plasmid and load onto a 0.8-2% TAE (Tris-acetate, not TBE) agarose gel (depending on the size of the band to be cut) and migrate at 80-90 volts until the bands can be easily cut. An example of the cloning of the hTERT in pENTR1A vector (w664-1, Addgene #) is shown on the next page in Figure 8.

7. Cut the band with a razor blade and determine its weight. The DNA will be isolated by Geneclean.

8. Add 3X volumes of NaI (i.e. 0.05 g band will need 150 ul of NaI).

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

10. 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.

11. 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).

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

13. Spin at maximum speed for 1 min. Keep supernatant and put it in the ligation tube. Ligate the plasmids as follows:

    10 ul of cDNA
    8 ul of Entry vector CIP treated
    2 ul 10X T4 DNA ligase buffer
    1 ul T4 DNA ligase

Incubate the ligations at 15°C overnight. Transform into a suitable *E.coli* strain. I normally use TOP10F' (Invitrogen).

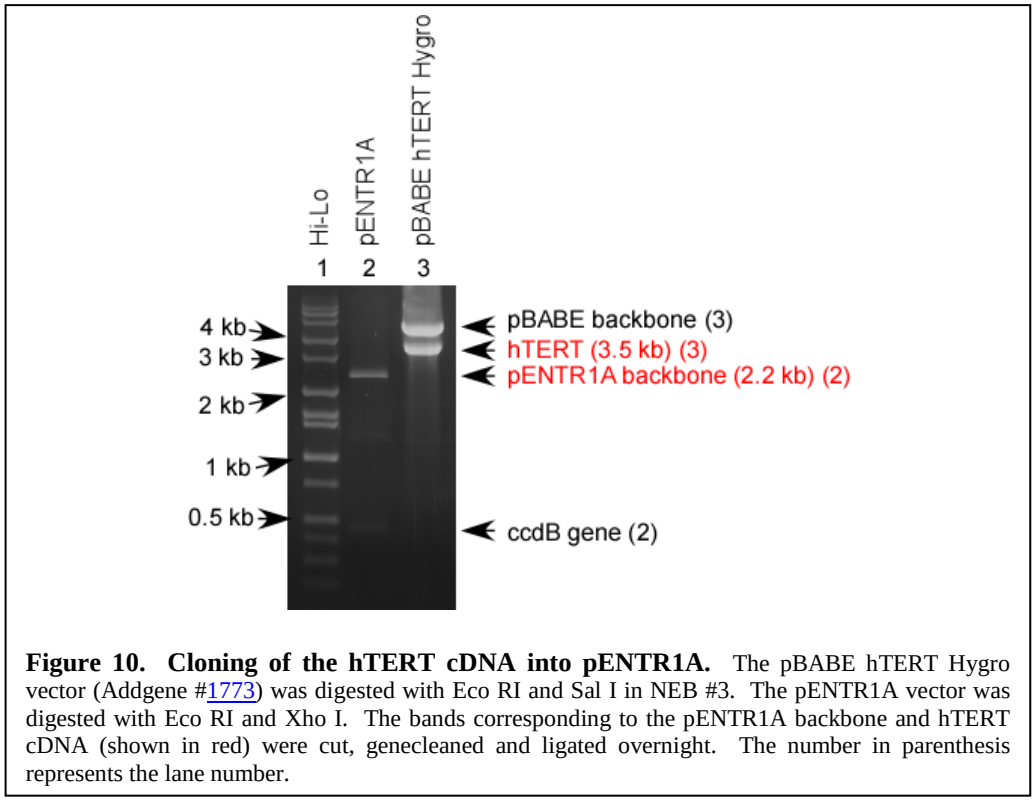


Figure 10. Cloning of the hTERT cDNA into pENTR1A. The pBABE hTERT Hygro vector (Addgene #1773) was digested with Eco RI and Sal I in NEB #3. The pENTR1A vector was digested with Eco RI and Xho I. The bands corresponding to the pENTR1A backbone and hTERT cDNA (shown in red) were cut, genecleaned and ligated overnight. The number in parenthesis represents the lane number.

Verification of the clones by restriction digest and sequencing. Pick colonies, isolate the plasmid by miniprep, and digest the plasmids to verify the presence of the insert. **Verify the clone by sequencing, to make sure no mistakes were made during the cloning or PCR step (if the cDNA was amplified by PCR).** I use the ENTRY forward and reverse sequencing primers most of the time (ENTRforw (5' to 3'): GCTCGGGCCCCAAATAATGA; ENTRrev (5' to 3'): CACGGGCCAGAGCTGCAGCTGGATGGCA). However, other primers from the fusion protein such as GFP or GST or the EF-1α promoter can also be used. Their sequences are given on page 39.

For the hTERT in pENTR1A vector (w664-1, Addgene #), plasmids were digested with Eco RI and Eco RV (the Sal I/Xho I ligation destroyed the Xho I site) and ran on a 1% TAE-agarose gel. The result of the agarose gel is shown in Figure 11 and of the sequencing in Figure 12:

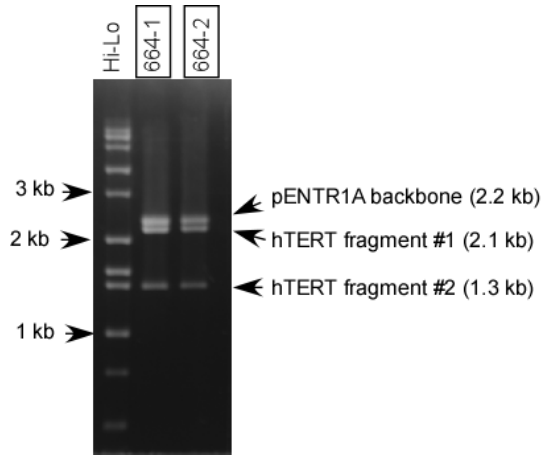


Figure 11. Eco RI/Eco RV digestion of clones 664-1 and 664-2. After transformation in TOP10F', two colonies were picked and grown in 2 ml of LB-Kan. 600 ul of the overnight culture was used for a Zippy miniprep and 10 ul was digested with Eco RI and Eco RV (Eco RV was used because the Xho I site was destroyed upon insertion of the hTERT cDNA and also because it is present in the hTERT cDNA and can be used to confirm the cDNA). Both clones are good. The 664-1 clone was sent for sequencing with the ENTRforw and ENTRrev primers.

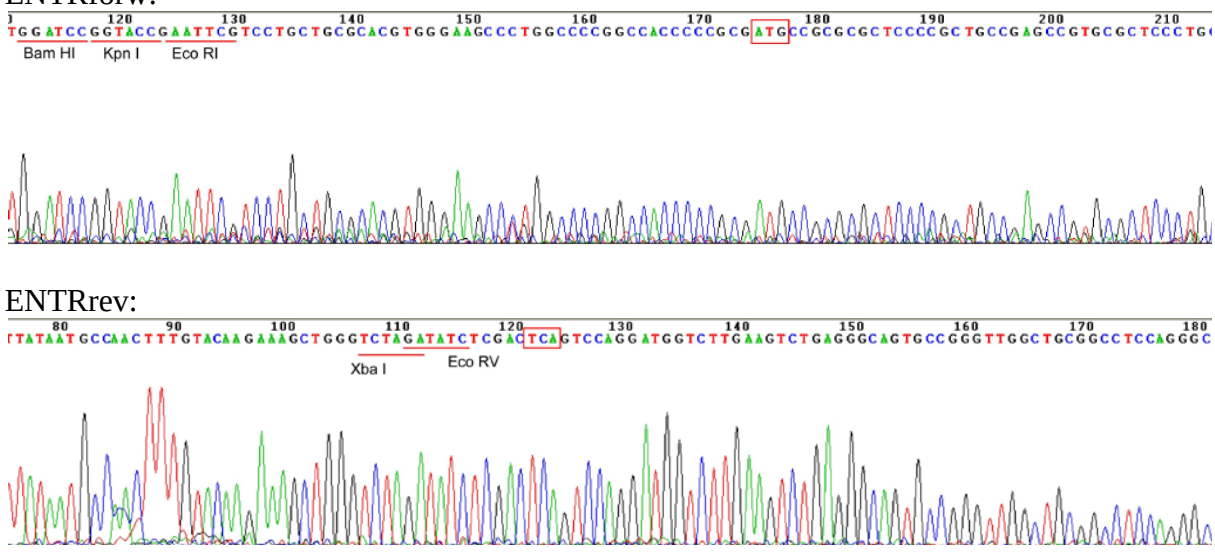


Figure 12. Confirmation of the hTERT in pENTR1A vector by sequencing. The vector was sequenced using the pENTRforw and pENTRrev primers. TOP: ENTRforw sequence. The 5' end of the multiple cloning site is seen as well as the translation initiation codon (ATG) of hTERT. BOTTOM: ENtrrev sequence. The translation termination codon (TGA) is seen as well as the 3' end of the multiple cloning site.

2- 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. Refer to Table 1 for the compatibilities between the Entry and Destination vectors.

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 cDNA: **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 (Addgene #17299, 668-1) and the pLenti CMV/TO Zeo (Addgene #17294, 644-1) 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, the pENTR/hTERT vector obtained previously was recombined with the pLenti CMV Neo DEST (705-1, Addgene #17392, ligation #665) and the pLenti PGK Blast vector (w524-1, Addgene #19065, ligation #666). The ligation reactions were transformed in the *E.coli* Stbl3 strain and plated on an LB ampicillin plate. The next day, one colony was picked for each transformation and grown overnight for a miniprep followed by digestion with Nco I and agarose electrophoresis. Figure 13 shows that both constructs are good. These constructs are not available through Addgene at the time of writing because they have not been fully characterized yet. The map and sequence of these vectors are available in the future vector section of the web site (here).

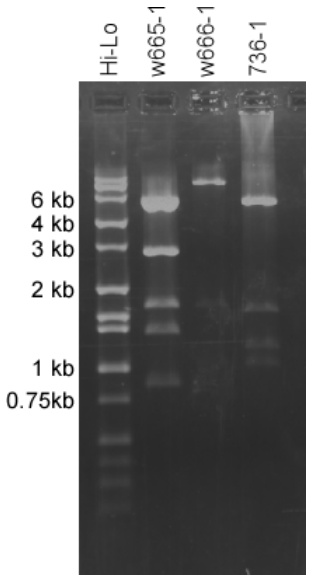


Figure 13. Screening of candidate clones after the LR recombination by restriction digest. One colony for each construct (#665, 666) was analyzed by Nco I restriction digest. Both colonies gave the expected restriction pattern. Plasmid 736-1 (pLenti CMV GFP DEST) was used as appositive control for the digestion.

Confirmation of the expression clone by sequencing

Sequencing the expression construct is not generally required. However, if you wish to confirm the expression clone, the following sequencing primers can be used: CMV forward primer: CGCAAATGGGCGGTAGGCGTG (do not use for QCXI series, there are two CMV promoters in these vectors); EF-1α: TCAAGCCTCAGACAGTGGTTC; EGFP-C: CATGGTCCTGCTGGAGTTCGTG (3' end of EGFP, forward primer); EGFP-N: CGTCGCCGTCCAGCTCGACCAG (5' end of EGFP, reverse primer); human PGKforw: GTAGTGTGGGCCCTGTTCCTGCC (not compatible with the mouse PGK promoter); Lucf: AGTCAAGTAACAACCGCGA (3' end of luciferase, forward primer, for sequencing miRNAs inserted after LUC); pGEX5': GGGCTGGCAAGCCACGTTTGGTG (3' end of glutathione-S-transferase, forward primer); T7: TAATACGACTCACTATAGGG (for sequencing 5' end of cDNAs under the control of the EF-1a promoter); V5 reverse: ACCGAGGAGAGGGTTAGGGAT (for sequencing 3'end of cDNAs under the control of the EF-1α promoter); WPRE reverse: CATAGCGTAAAAGGAGCAACA (5' end of WPRE, reverse primer, for sequencing 3' end of cDNAs).

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). This protocol describes the use of the Qiafilter midi prep kit.
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]. During the centrifugation, prepare the QIAfilter Cartridge[2]: Screw the cap onto the outlet nozzle of the QIAfilter Midi or QIAfilter Maxi Cartridge. Place the QIAfilter Cartridge in a convenient tube.
6. After the centrifugation, pour the supernatant into the barrel of the QIAfilter Cartridge. Incubate 5 minutes at room temperature.
7. Meanwhile, equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT and allow the column to empty by gravity flow.
8. Remove the cap from the QIAfilter Cartridge outlet nozzle. Gently insert the plunger into the QIAfilter Midi Cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip.
9. Allow the cleared lysate to enter the resin by gravity flow.
10. Wash the QIAGEN-tip with 2 x 10 ml of Buffer QC. Transfer the QIAGEN-tip over a 50 ml centrifuge tube.
11. Elute the plasmid DNA with 5 ml of Buffer QF.
12. 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.
13. 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.
14. Air-dry the pellet for 5–10 min, and redissolve the DNA in 100 µl of water.
15. Determine the DNA concentration by UV spectrophotometry

NOTES:

[1]The Qiagen manual does not suggest a centrifugation at this step for the Qiafilter preps. 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.

[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.

3A- Producing Lentiviruses in 293T Cells

To produce **lentiviruses**, three packaging plasmids will be co-transfected with the vector containing the cDNA. We 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 requires special care. After the transfection, 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).

3B- 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).

4-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.

5- Transduction and expression of the cDNA

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 the cDNA, 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 cDNA.

Clonal isolates or population?

Depending on the efficiency of the T-REx cell line, it might be necessary to isolate colonies in order to obtain minimal leakiness and maximal induction of the protein. It is recommended to freeze-down aliquots of the cells as early as feasible, especially if overexpression of the protein is deleterious to the cell. In this case, subpopulations with lower levels of cDNA have a growth advantage and slowly take over the culture. This difference in cDNA levels could be due to either the number of viruses integrated in the host genome, the genomic location of the viral insertion (active versus inactive chromatin), or methylation of the promoter driving the cDNA expression. For inducible expression of a 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 [5]. However, we observed cases where we could not generate an inducible cell line. We postulated it could be due to either the high toxicity of the protein to be expressed, or to improper regulation of the protein when taken out of its genomic context (transcription, splicing or other post-transcriptional regulations).

Time for optimal expression.

It is recommended to do a time-course for the overexpression of the protein of interest to determine the time of maximal expression. The maximal expression is normally attained at 48 hours post-induction or post-transduction, although results might vary depending on the protein. The promoter driving overexpression of the cDNA might get methylated and at longer time points, the overexpressed protein might no longer be detectable.

FKBP-fused proteins.

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 [5]. Please refer to the background section 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 expression 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 [38].

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 cDNA 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
Overexpression of a protein can be detrimental to the cell due to sequestering of the transcription/translation machinery or accumulation of proteins in the cell. We always express a "neutral" cDNA (either the firefly luciefrase or GFP) as a negative control in our experiments to account for such an effect. Some Entry vectors and recombined Destination vectors were generated for both LUC and GFP and they were sent to Addgene. Their maps can be found in the appendix.

Materials required
• A viral stock of the virus overexpressing the cDNA
• A host cell line (T-REx for inducible depletion)
• 6 mg/ml Polybrene®
• 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 expression:</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 expression. The next day, add doxycycline to one well at a final concentration of 1 ug/ml and incubate for 48h.

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

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.

For some examples of overexpression using the various vectors, please refer to the article describing these vectors [5].

VI-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)}f *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 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 virus expressing the cDNA
- HeLa or HT1080 cells
- 6 mg/ml Polybrene®
- 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 14.

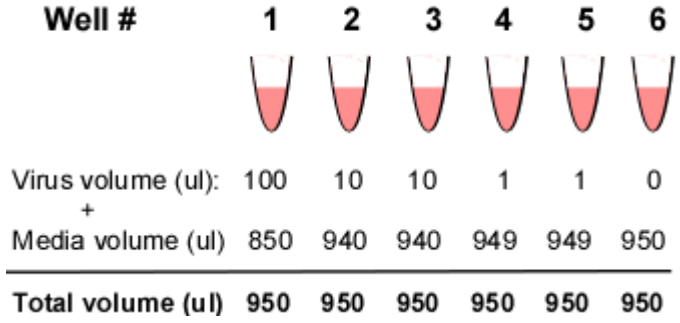


Figure 14. Titering of the viral stock in a six-well plate. Different amounts of viral supernatants are added in tubes 1-6. Media containing polybrene (6ug/ml) is added to each tube for a final volume of 950ul.

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 15. Incubate overnight in the incubator.

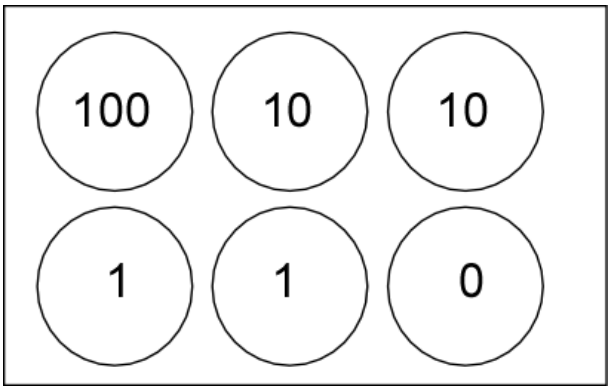


Figure 15. Layout of the viral titering plate. For each well, the number represents the amount of viral supernatant originally added to each tube.

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 <u>live and functional</u> 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 dd$H_2$0 (normally, dunk twice in cold water).
8. Allow plates to dry at room temperature (may take overnight).

A typical result is shown in Figure 16 for the pQCXIN X2/pTER shLUC plasmid (w347-1, Addgene #17489).

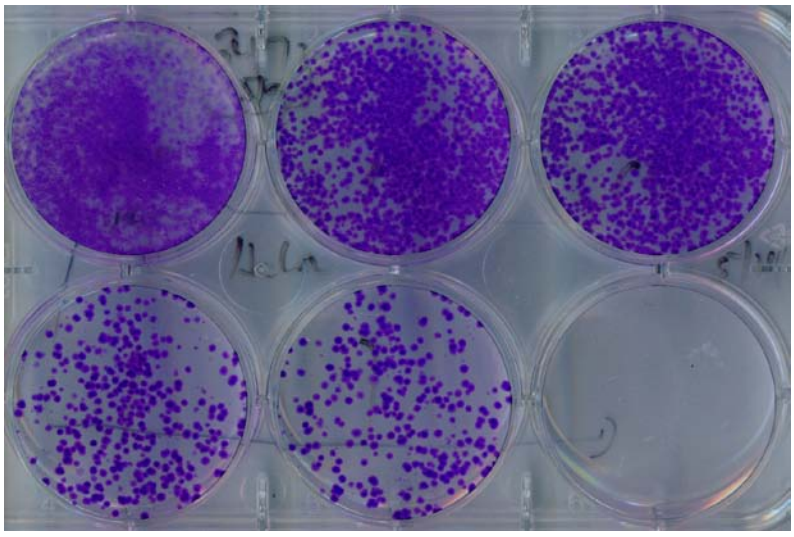


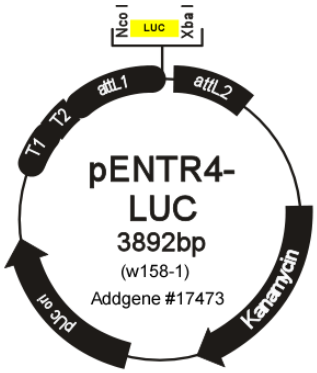
Figure 16. Titration of the pQCXIN X2/pTER shLUC plasmid. The pENTR/pTER shLUC (w177-1, Addgene #17472) was recombined with the pQCXIN X2 DEST vector (w310-1, Addgene #17399) to generate the pQCXIN X2/pTER shLUC plasmid. The plasmid was transfected in 293Gag-pol cells with the pGAG-pol and pVSV-G packaging 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 x10^{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 control vectors for expression studies

A) Firefly luciferase vectors

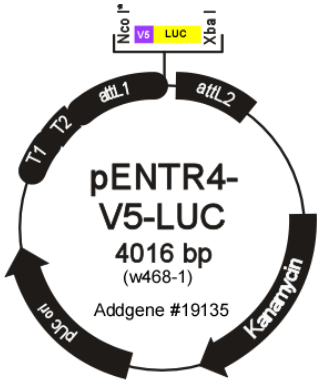
pENTR-LUC (w158-1, Addgene #17473):



Comment s for pENTR 4-LUC
3892 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457
Firefly luciferase (LUC): bases 463-2115
attL2: bases 2122-2222
Kanamycin resistance gene: bases 2344-3153
pUC origin: bases 3219-3891

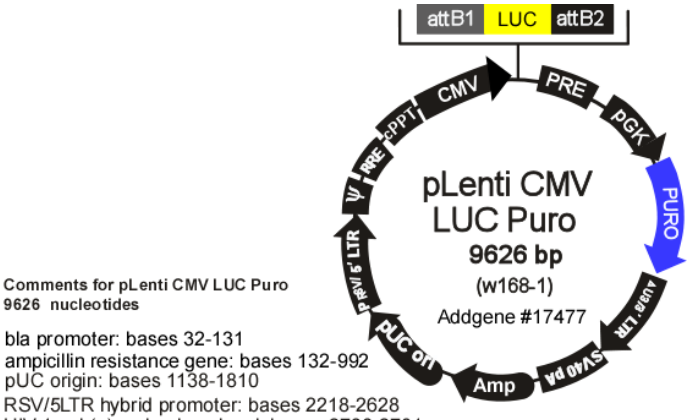
pENTR4-V5-LUC (w468-1, Addgene #19135):



Comment s for pENTR 4 V5-LUC
4016 nucleotides

rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1:bases 358-457
V5 epitope: bases 463-511
Firefly luciferase (LUC): bases 586-2238
attL2: bases 2245-2345
Kanamycin resistance gene: bases 2418-3276
pUC origin: bases 3342-4014

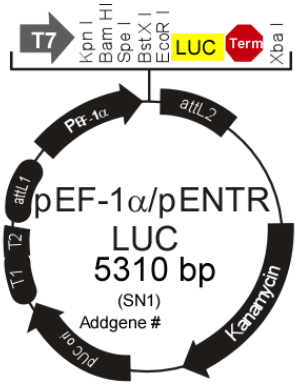
pLenti CMV LUC Puro (w168-1, Addgene #17477):



Comments for pLenti CMV LUC Puro
9626 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
CMV promoter: bases 4145-4660
*att*B1 site: bases 4764-4788
Firefly luciferase (LUC): bases 4794-6446
*att*B2 site: bases 6453-6477
Woodchuck post-transcriptional element (PRE): bases 6507-7099
Murine phosphoglycerate kinase (pGK) promoter: bases 7100-7610
Puromycin resistance gene: bases 7630-8229
ΔU3/3'LTR: bases 8366-8600
SV40 polyadenylation signal: bases 8669-8824

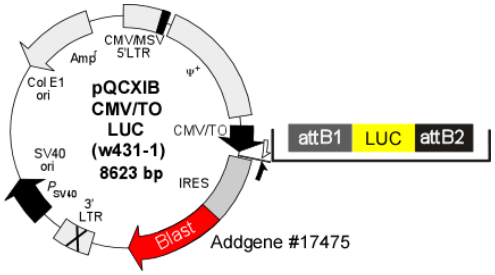
pEF-ENTR LUC (SN1, Addgene #22524):



rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
Elongation Factor 1 alpha (EF-1a) promoter: bases 614-1770
Firefly Luciferase (LUC) cDNA: bases 1881-3533
attL2:bases 3540-3640
Kanamycin resistance gene: bases 3762-4571
pUC origin: bases 4680-5307

This vector was made by Stefania Nicoli (University of Massachusetts Medical School).

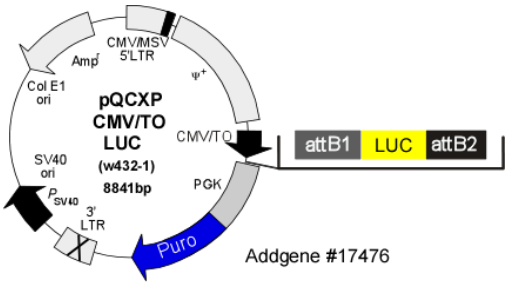
pQCXIB CMV/TO LUC (w431-1, Addgene #17475):



Comments on pQCXIB CMV/TO LUC, 8623 nucleotides:

5' LTR (CMV/MSV): 1–728
Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter: 1–511
R region: 584–654
U5 region: 655–728
ψ+ (extended packaging signal): 758–1567
CMV/TO promoter: 1601-2170
attB1:2198-2222
Firefly Luciferase (LUC): bases 2228-3880
attB2:3887-3911
Internal ribosome entry site (IRES): 4052-4626
Blasticidin resistance gene (Blast): 4683-5081
3' MoMuLV LTR (deletion in U3): 5329-5754
SV40 promoter: 6033-6300
SV40 ori: 6255-6320
Col E1 ori (Site of replication initiation): 6642
Ampicillin resistance gene (reverse orientation): 8261-7401

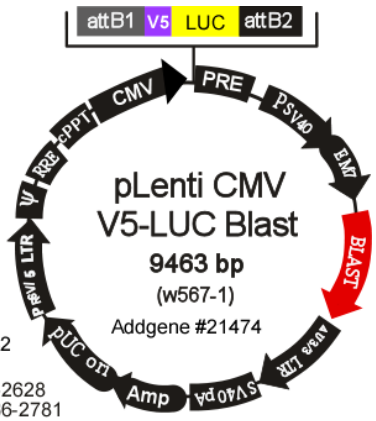
pQCXP CMV/TO LUC (w432-1, Addgene #17476):



Comments on pQCXP CMV/TO LUC, 8841 nucleotides:

5' LTR (CMV/MSV): 1–728
Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter: 1–511
R region: 584–654
U5 region: 655–728
ψ+ (extended packaging signal): 758–1567
CMV/TO promoter: 1601-2159
attB1:2198-2222
Firefly Luciferase (LUC): bases 2228-3880
attB2:3887-3911
Murine phosphoglycerate kinase (PKG) promoter:4050-4551
Puromycin resistance gene (Puro): 4574-5173
3' MoMuLV LTR (deletion in U3): 5547-5972
SV40 promoter: 6251-6518
SV40 ori: 6473-6538
Col E1 ori (Site of replication initiation): 6860
Ampicillin resistance gene (reverse orientation):8479-7619

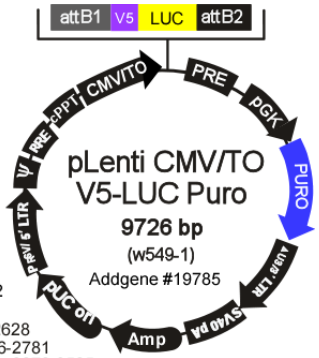
pLenti CMV V5-LUC Blast (w567-1, Addgene #21474):



comments for pLenti CMV V5-LUC Blast
9463 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
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
CMV promoter: bases 4145-4730
*att*B1 site: bases 4763-4790
V5-LUC: bases 4793-6569
*att*B2 site: bases 6576-6600
Woodchuck post-transcriptional element (PRE): bases 6630-7223
SV40 early promoter and origin: bases 7287-7650
EM7 promoter: bases 7653-7718
Blasticidin resistance gene: bases 7719-8117
ΔU3/3'LTR: bases 8204-8437
SV40 polyadenylation signal: bases 8508-8661

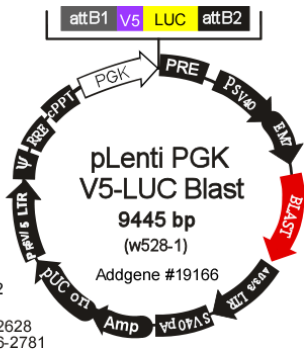
pLenti CMV/TO V5-LUC Puro (w549-1, Addgene #19785):



Comments for pLenti CMV/TO V5-LUC Puro
9726 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
CMV/TO promoter: bases 4145-4660
*att*B1 site: bases 4764-4788
V5 epitope: bases
Firefly luciferase (LUC): bases
*att*B2 site: bases
Woodchuck post-transcriptional element (PRE): bases
Murine phosphoglycerate kinase (pGK) promoter: bases
Puromycin resistance gene: bases
ΔU3/3'LTR: bases
SV40 polyadenylation signal: bases

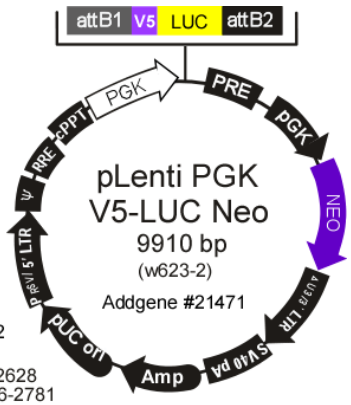
pLenti PGK V5-LUC Blast (w528-1, Addgene #19166):



comments for pLenti PGK V5-LUC Blast
9445 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
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
Phosphoglycerate kinase (PGK) promoter: bases 4180-4695
*att*B1 site: bases 4746-4770
V5 epitope: bases 4776-4823
Firefly Luciferase (LUC): bases 4830-6551
*att*B2 site: bases 6558-6582
Woodchuck post-transcriptional element (PRE): bases 6612-7205
SV40 early promoter and origin: bases 7253-7595
EM7 promoter: bases 7635-7700
Blasticidin resistance gene: bases 7701-8099
ΔU3/3'LTR: bases 8187-8419
SV40 polyadenylation signal: bases 8490-8643

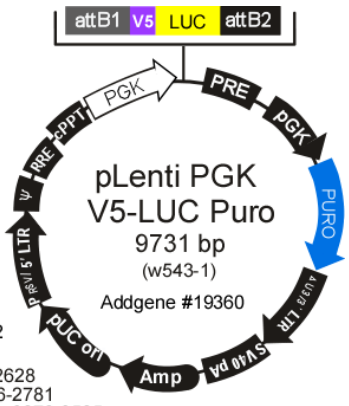
pLenti PGK V5-LUC Neo (w623-2, Addgene #21471):



Comments for pLenti PGK V5-LUC Neo
9910 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
Human phosphoglycerate kinase (PGK) promoter: bases 4179-4694
*att*B1site: bases 4745-4770
V5-LUC: bases 4776-6551
*att*B2 site: bases 6558-6582
Woodchuck post-transcriptional element (PRE): bases 6612-7205
Murine phosphoglycerate kinase (pGK) promoter: bases 7235-7743
Neomycin resistance gene: bases 7757-8560
ΔU3/3'LTR: bases 8651-8884
SV40 polyadenylation signal: bases 8955-9108

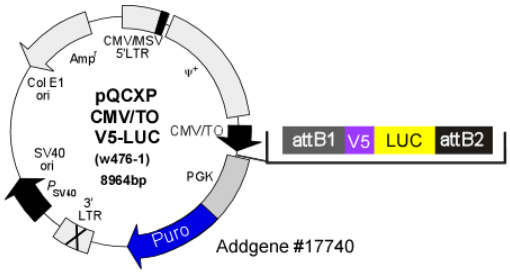
pLenti PGK V5-LUC Puro (w543-1, Addgene #19360):



Comments for pLenti PGK V5-LUC Puro
9731 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
Human phosphoglycerate kinase (PGK) promoter: bases 4179-4694
*att*B1site: bases 4745-4770
V5-LUC: bases 4776-6551
*att*B2 site: bases 6558-6582
Woodchuck post-transcriptional element (PRE): bases 6612-7205
Murine phosphoglycerate kinase (pGK) promoter: bases 7210-7714
Puromycin resistance gene: bases 7735-8334
ΔU3/3'LTR: bases 8473-8705
SV40 polyadenylation signal: bases 8776-8929

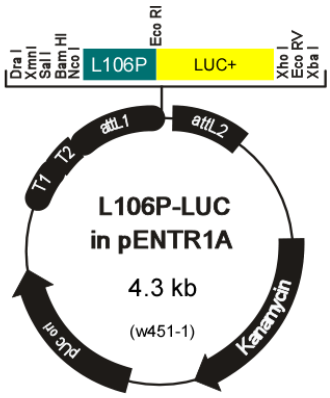
pQCXP CMV/TO V5-LUC (w476-1, Addgene #17740):



Comments on pQCXP CMV/TO V5-LUC, 8964 nucleotides:

5' LTR (CMV/MSV): 1–728
Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter: 1–511
R region: 584–654
U5 region: 655–728
ψ+ (extended packaging signal): 758–1567
CMV/TO promoter: 1601-2159
attB1:2198-2222
V5 epitope: bases 2228-2272
Firefly Luciferase (LUC): bases 2351-4003
attB2:4010-4034
Murine phosphoglycerate kinase (PKG) promoter:4173-4674
Puromycin resistance gene (Puro): 4697-5296
3' MoMuLV LTR (deletion in U3): 5670-6095
SV40 promoter: 6374-6641
SV40 ori: 6596-6661
Col E1 ori (Site of replication initiation): 6983
Ampicillin resistance gene (reverse orientation):8602-7742

pL106P-Luc in pENTR1A (w451-1, Addgene #17455):

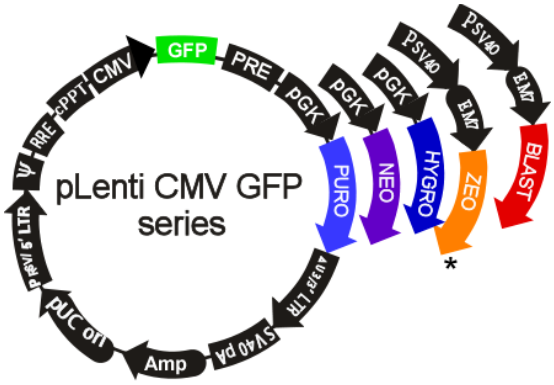


Comments for L106P-LUC in pENTR1A
4262 nucleotides

rrnBT1 transcription termination sequence: bases 106-149
rrnBT2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
FKBP L106P domain: bases 494-817
Firefly luciferase ORF: bases 824-2476
attL2: bases 2491-2589
Kanamycin resistance gene: bases 2713-3522
pUC origin: bases3586-4259

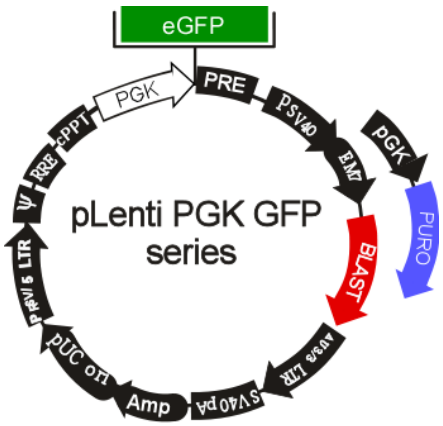
B) Green Fluorescent Protein (GFP) Vectors

pLenti CMV GFP series:



Blast:659-1, Addgene #17445
Hygro: 656-4, Addgene #17446
Neo: 657-2, Addgene #17447
Puro: 658-5, Addgene #17448
Zeo*:637-7, Addgene #17449

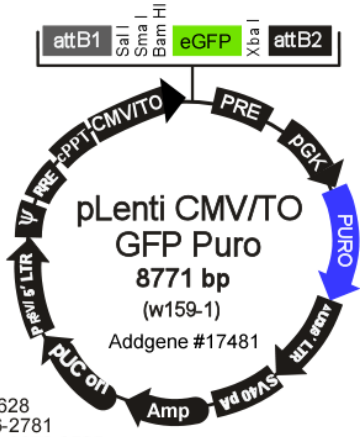
pLenti PGK GFP series:



Blast: w510-5, Addgene #19069
Puro: w509-5, Addgene #19070

159-1: pLenti CMV/TO eGFP Puro
301-1: GFP in pQCXIB

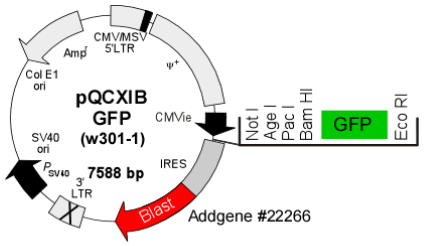
pLenti CMV/TO GFP Puro (w159-1, Addgene #17481):



Comments for pLenti CMV/TO GFP Puro
8771 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
CMV/TO promoter: bases 4145-4702
*att*B1 site: bases 4764-4788
Enhanced green fluorescent protein (eGFP): bases:4854-5573
*att*B2 site: bases 5598-5622
Woodchuck post-transcriptional element (PRE): bases 5652-6245
Murine phosphoglycerate kinase (pGK) promoter: bases 6246-6755
Puromycin resistance gene: bases 6775-7374
ΔU3/3′LTR: bases 7513-7745
SV40 polyadenylation signal: bases 7816-7969

pQCXIB GFP (w301-1, Addgene #22266):

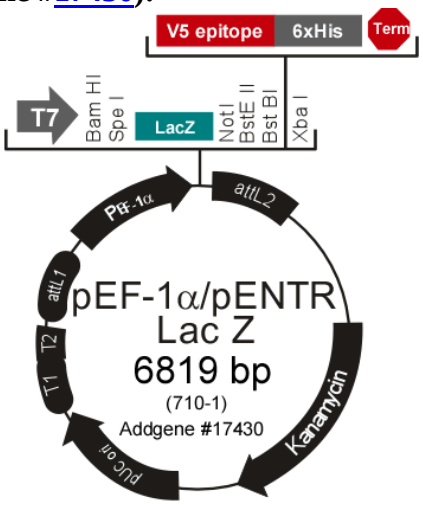


Comments on pQCXIB GFP, 7588 nucleotides:

5' LTR (CMV/MSV): 1–728
Cytomegalovirus (CMV)/ mouse sarcoma virus (MSV) hybrid promoter: 1–511
R region: 584–654
U5 region: 655–728
ψ+ (extended packaging signal): 758–1567
CMV promoter: 1601-2133
Enhanced Green Fluorescent Protein (GFP) cDNA: bases 2288-3007
Internal ribosome entry site (IRES): 3016-3588
Blasticidin resistance gene (Blast): 3648-4074
3' MoMuLV LTR (deletion in U3): 4294-4720
SV40 promoter: 4998-5265
SV40 ori:5220-5286
Col E1 ori (Site of replication initiation): 5606
Ampicillin resistance gene (reverse orientation):7226-6366

C) β-galactosidase (Lac Z) vector

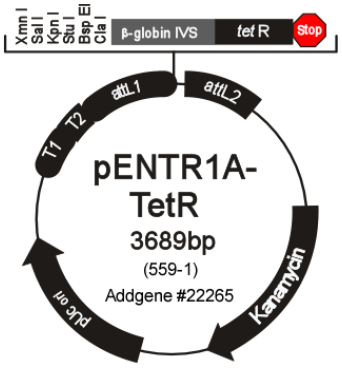
pEF-ENTR-LacZ (710-1, Addgene #17430):



rrnB T1 transcription termination sequence: bases 106-149
rrnB T2 transcription termination sequence: bases 281-308
attL1: bases 358-457 (complementary strand)
Elongation Factor 1 alpha (EF-1a) promoter: bases 614-1766
Lac Z ORF: bases 1883-4939
V5 tag: 4967-5006
6xHis: bases 5018-5035
Translation termination codon: bases 5036-5038
attL2: bases 5050-5150
Kanamycin resistance gene: bases 5271-6080
pUC origin: bases 6189-6816

D) Other vector:

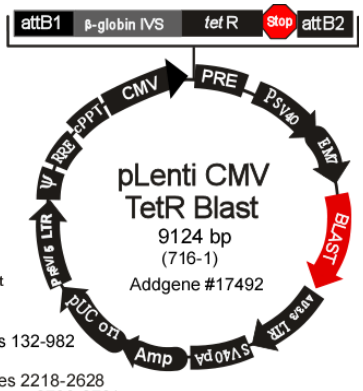
TetR in pENTR1A (559-1, Addgene #22265)



Comment s for pENTR1A-TetR
3689 nucleotides

rrnBT1 transcription termination sequence: bases 106-149
rrnBT2 transcription termination sequence: bases 281-308
attL1: bases 358-457
Rabbit β-globin intron II (IVS): bases 602-1174
Tetracycline repressor (TetR) gene: bases 1258-1905
attL2: bases 1919-2019
Kanamycin resistance gene: bases 2141-2950
pUC origin: bases 3060-3687

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



Comments for pLenti CMV TetR Blast
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

These vectors can be used to make T-REx cell lines. They have been used successfully in primary as well as transformed cell lines.

7) 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.

8) 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.

9) 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^{o}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^{o}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^{o}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^{o}C.
- Plate everything (ligation) or 25 µl (purified plasmid) on LB agar plates with appropriate antibiotic selection.
- Incubate at 37^{o}C overnight.

VII-References

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