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Trans-Lentiviral™ Packaging System

The safest lentiviral system for expression

TLP4614 - Trans-Lentiviral Packaging System, shRNA
TLP4615 - Trans-Lentiviral Packaging System, shRNA (contains cell line)

Introduction

The Trans-Lentiviral Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus which can be used to deliver and express your gene or shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes, *et al*. (2001).

System Components

The Trans-Lentiviral Packaging System includes the following components as listed in Table 1.

Table 1. Trans-Lentiviral Packaging System Components.

Product	Catalog no. *	Quantity
Trans-Lentiviral Packaging Mix		225µg
TLA-HEK293T™ Packaging Cell Line	HCL4517	1ml
Arrest-In™ Transfection Reagent	ATR1741	0.5ml x 4
pGIPZ™ Non-Silencing Control Vector		20µg (0.45µg/µl)
pGIPZ Cloning Vector		10µg (0.25µg/µl)

*For components that can be ordered individually.

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Shipping/Storage

The Trans-Lentiviral Packaging System is shipped as described below. Upon receipt, store each component as detailed below as listed in Table 2.

Table 2. Trans-Lentiviral Packaging System Shipping/Storage Conditions.

Item	Shipping	Storage
Trans-Lentiviral Packaging Mix	Blue ice	-20°C
TLA-HEK293T Packaging Cell Line	Dry ice	Liquid nitrogen
Arrest-In Transfection Reagent	Blue ice	+4°C
pGIPZ Non-Silencing Control Vector	Blue ice	-20°C
pGIPZ Cloning Vector	Blue ice	-20°C

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TLA-HEK293T™ Cell Line (optional)

In addition to the reagents provided in the Trans-Lentiviral™ Packaging System, some configurations contain the TLA-HEK293T packaging cell line. The TLA-HEK293T cell line is supplied as one vial containing ~1 x 10^6 frozen cells in 1ml of freezing medium. **Upon receipt, store in liquid nitrogen.**

BEWARE: ALWAYS WEAR PROTECTIVE EYEWEAR WHEN HANDLING VIALS STORED IN LIQUID NITROGEN.

The TLA-HEK293T cell line in this order has been shipped on dry ice. While precautions in packing have been taken to prevent CO_2 from entering the vial, it is suggested that the vial be stored for two days in liquid nitrogen upon receipt of the order to allow any CO_2 to dissipate. Also, as there is always potential of bursting, it is recommended that when the vial is removed from liquid nitrogen, it is left standing at room temperature for approximately 30 seconds to allow the liquid nitrogen to dissipate from the vial.

For instructions to thaw, culture, and maintain the TLA-HEK293T cell line, see pages 17-19.

Components of the Trans-Lentiviral System

The Trans-Lentiviral Packaging System possesses features which enhance its biosafety while allowing high-level gene or shRNA expression in a wider range of cell types than traditional retroviral systems. The system includes the following major components:

- The Trans-Lentiviral Packaging Mix contains an optimized mixture of the five packaging plasmids, pTLA1-Pak, pTLA1-Enz, pTLA1-Env, pTLA1-Rev and pTLA1-TOFF. These plasmids supply the helper functions as well as structural and enzymatic proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see pages 24-30.

- An optimized TLA-HEK293T producer cell line allows production of the lentivirus following co-transfection of the transfer plasmid and the packaging plasmids in the packaging mix. The cell line stably expresses the SV40 large T antigen and facilitates the production of high viral titers. *Optional – Not included in all configurations*

- A transfer vector (e.g. pGIPZ™ Non-Silencing) into which a TurboGFP reporter is cloned. The vector also contains the elements required to allow packaging of the expression construct into virions (e.g. 5′ and 3′ LTRs, Ψ packaging signal).

Co-transfection of the Trans-Lentiviral Packaging Mix and the transfer vector containing the gene of interest into TLA-HEK293T cells will produce a replication-incompetent lentivirus, which can then be transduced into the mammalian cell line of interest.

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Biosafety Features of the System

The Trans-Lentiviral™ Packaging System is based on lentiviral vectors developed by Kappes and Wu (2,3,5,6). This newest generation lentiviral system includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus. More significantly, the reverse transcriptase (RT) and integrase (IN) proteins are split from the native gag-pol polyprotein structure and are provided *in trans* from a separate plasmid producing a novel class of HIV-based vectors. Instead of expressing Gag-Pol, the Trans-Lentiviral system contains a plasmid that expresses Gag/Gag-Pro and one that expresses Pol (RT & IN) fused to Vpr. Removing the RT & IN from the packaging construct prevents the lentiviral replication machinery from functioning. This system, in contrast to the standard third generation vectors, prevents the generation of recombinant viral particles that possess the required functional gag-pol structure for DNA mobilization and the emergence of replication competent lentivirus. Figure 1 illustrates the absence of functional gag-pol recombinants in lentiviral stocks generated from the Trans-Lentiviral Packaging System.

Use of the Trans-Lentiviral Packaging System to facilitate lentiviral-based expression of the gene of interest provides the following advantages:

- Generates an HIV-1-based lentivirus vector that effectively transduces both dividing and non-dividing mammalian cells.
- Efficiently delivers the gene of interest to mammalian cells in culture or *in vivo*.
- Eliminates the production of replication competent viral particles.

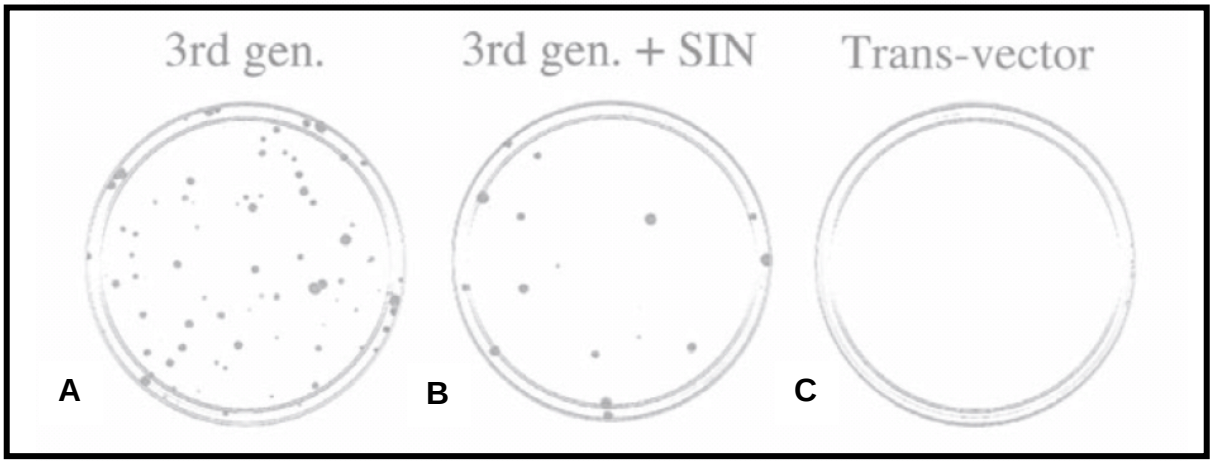

Figure 1. Stocks of lentiviral vectors were prepared using a third generation packaging construct (A), a third generation packaging construct in combination with a SIN vector (B), and the *trans*-vector (C), respectively. 10^8 infectious units of A and B and 10^9 infectious units of C were used to infect cultures of 293T cells containing the puromycin resistance gene. Three days later the culture supernatants were collected, concentrated by ultracentrifugation and used to infect HeLa-tat cells. After selecting in medium containing puromycin the resistant cell colonies were visualized by staining with crystal violet. *Kappes & Wu, Somatic Cell and Molecular Genetics, Vol. 26, Nos. 1/6, November 2001.*

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The Trans-Lentiviral™ Packaging System includes the following key safety features:

- The expression vectors contain a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Zufferey *et al*., 1998).
- The number of genes from HIV-1 that are used in the system has been reduced (i.e. *gag*, *pol*, *rev, tat* and *vpr*).
- The VSV-G gene from Vesicular Stomatitis Virus is used to pseudotype the vector particles (Yee *et al*., 1994). The HIV-1 envelope has been completely removed from the vector.
- Genes encoding the structural and other components required for packaging the viral genome are separated onto four plasmids.
- Although the packaging plasmids allow expression *in trans* of genes required to produce viral progeny (e.g. *gag, pol, rev, tat, env*) in the TLA-HEK293T™ producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus has been shown to be produced.

Biosafety Level 2

For more information regarding viral agents and Biosafety Level 2 laboratory guidelines and precautions, please refer to the links below:

NIH Agent Summary Statement: http://bmbl.od.nih.gov/viral2.htm#retro

NIH Biosafety Level 2 Description: http://bmbl.od.nih.gov/sect3bsl2.htm

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Trans-Lentiviral™ Packaging Mix

The pTLA1-Pak, pTLA1-Enz, pTLA1-Rev, pTLA1-Env, pTLA1-TOFF plasmids are provided in an optimized mixture to facilitate viral packaging of the transfer vector following co-transfection into TLA-HEK293T™ producer cells. The amount of the packaging mix and transfection reagent supplied in the Trans-Lentiviral Packaging Mix is sufficient to perform 10 packaging events in 100mm plates.

TLA-HEK293T Cell Line

The human TLA-HEK293T cell line is supplied with the Trans-Lentiviral Packaging System to facilitate optimal lentivirus production. The TLA-HEK293T cell line stably and constitutively expresses the SV40 large T antigen.

For optimal lentivirus production (i.e. producing lentiviral stocks with the expected titers), follow the guidelines on pages 17-18 to culture TLA-HEK293T cells before use in transfection.

Control Plasmids

Each Trans-Lentiviral Packaging System includes a control vector for use as an expression control (pGIPZ™ Non-Silencing). We recommend including the control vector in the co-transfection procedure to generate a control lentiviral stock that may be used to help optimize expression conditions in the mammalian cell line of interest.

Transfection Reagent

Arrest-In™ transfection reagent is a proprietary lipo-polymeric formulation, developed and optimized for transfection of shRNA plasmid DNA into the nucleus of cultured eukaryotic cells. Arrest-In also provides an enhanced uptake efficiency of the shRNA plasmid DNA into cells.

Producing Lentivirus in TLA-HEK293T Cells

Before a stably transduced cell line expressing the gene or shRNA of interest can be created, a lentiviral stock (containing the packaged transfer vector) will first need to be produced by co-transfecting the optimized packaging plasmid mix and the transfer vector construct into the TLA-HEK293T cell line. The following section provides protocols and instructions to generate a lentiviral stock.

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Recommended Transfection Conditions

Produce lentiviral stocks in TLA-HEK293T™ cells using the following optimized transfection conditions below. The amount of lentivirus produced using these recommended conditions generally results in a titer of approximately 1 x 10^5 – 1 x 10^6 transducing units (TU) per ml (unconcentrated). We recommend concentrating the viral stock to obtain a titer of approximately 1 x 10^8 transducing units (TU) per ml.

Table 3. Transfection Components.

Components	Amount
Tissue culture plate size	100mm (one per lentiviral construct)
Number of TLA-HEK293T cells to transfect	5.5 x 10^6 cells
Amount of Trans-Lentiviral™ Packaging Mix	22.5µg (21µl of packaging mix stock)
Amount of pGIPZ™ transfer vector	9µg
Amount of Arrest-In™ transfection reagent	158µg (158µl of 1mg/ml stock)

Arrest-In Transfection Reagent for Delivery of shRNA

It is preferable that transfection be carried out in medium that is serum free and antibiotic free.

The protocol below is optimized for transfection of the packaging mix and transfer vector construct into TLA-HEK293T cells in a 100mm plate. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area.

Warm Arrest-In to ambient temperature (approximately 20 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use.

Maintain sterile working conditions with the DNA and Arrest-In mixtures as they will be added to the cells.

Table 4. Suggested amounts of DNA, medium and transfection reagent to deliver constructs into adherent cells.

Tissue Culture Dish	Surface area per well (cm^2)	Total serum free medium volume per well (ml)	Packaging mix & Transfer vector plasmid DNA (µg)*	Transfection Reagent (µl)**
100 mm	56	5ml	31.5µg	158µl

*Recommended starting amount of DNA. May need to be optimized for the highest efficiency.
**Recommended starting amounts of transfection reagent.

1. The day before transfection (day 0), plate the TLA-HEK293T cells at a density of 5.5 x 10^6 cells per 100mm plate.

   Full medium (i.e. with serum and antibiotics) will still be used at this stage.

2. On the day of transfection form the DNA/Arrest-In complexes.

   The principle is to prepare the DNA and transfection reagent dilutions in an equal amount of serum-free medium in two separate tubes. These two mixtures (i.e. the DNA and the

Arrest-In) will be added to each other and incubated for 20 minutes prior to addition to the cells. This enables the DNA/Arrest-In complexes to form.

Allow the Arrest-In™ to come to room temperature. Mix the transfection reagent thoroughly by vortexing before beginning.

Dilute 31.5µg DNA into 1ml (total volume) of serum-free medium in a tube. Dilute 158µl of Arrest-In into 1ml (total volume) of serum-free medium.

This will give a 1:5 DNA:Arrest-In ratio which is recommended for successful transfection.

Add the 1ml of medium containing the diluted Arrest-In to the 1ml diluted DNA, mix well and incubate at room temperature for 20 minutes to form the transfection complexes.

The total volume will be 2ml at this stage.

3. Aspirate the growth medium from the cells. Add an additional 3ml of serum-free medium to each of the tubes containing the transfection complexes, mix gently, then overlay onto the cells. Return the cells to the CO_2 incubator at 37°C for 3-6 hrs.

   The total volume will be 5ml at this stage.

4. Aspirate the transfection mixture and replace with 12ml standard culture medium. Return the cells to the CO_2 incubator at 37°C.

5. After 48-72 hours of incubation, examine the cells microscopically for the presence of TurboGFP expression which is an indication of transfection efficiency.

   Factors affecting transfection efficiency are not limited to but include purity of DNA, health of transfected cells, inconsistencies in number of cells plated, insufficient mixing of transfection complexes.

Additional Factors Influencing Successful Transfection:

a. **Concentration and purity of nucleic acids**

b. **Transfection in serum-containing vs. serum-free medium** – Our studies indicate that Arrest-In/DNA complexes should preferably be formed in the absence of serum. In the cell lines tested, the highest transfection efficiencies can be obtained if the cells are exposed to the transfection complexes in serum-free conditions.

c. **Cell history, density, and passage number** – It is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before. However, adequate time should be given to allow the cells to recover from the passaging (generally >12 hours). If transfection efficiencies are low or reduction occurs over time, thawing a new batch of cells or using cells with a lower passage number may improve the results.

Note: Expression of the VSV-G glycoprotein causes HEK293T cells to fuse, resulting in the appearance of multinucleated syncitia. This morphological change is normal and does not affect production of the lentivirus.

6. Harvest virus-containing supernatants 48-72 hours post-transfection by removing medium to a 15ml sterile, capped, conical tube.

   If you are concentrating virus, you should harvest the supernatant at two intervals, 48 and 72 hours post-transfection. At 48 hours post-transfection, collect the supernatant into a 50ml sterile, capped, conical tube. Store the tube containing supernatant overnight at 4ºC. To the 100-mm plate, carefully pipette 12ml standard culture medium back onto the transfected cells and return plate to the CO_2 incubator at 37°C. The following day (72 hours post-transfection), harvest the culture supernatant and combine with supernatant harvested the day before. Follow the protocol below for concentration of the virus stock.

   ***Caution:** Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see page 4 for more information).*

7. Centrifuge tube containing harvested supernatant at 3000 rpm for 20 minutes at 4°C to pellet cell debris. Perform filtration step*, if desired.

   **If you plan to use your lentiviral construct for in vivo applications, we recommend filtering your viral supernatant through a sterile, 0.45µm low protein binding filter after the low-speed centrifugation step to remove any remaining cellular debris. We recommend using Millex-HV 0.45µm PVDF filters (Millipore, Catalog no. SLHVR25LS) for filtration.*

   We recommend that you concentrate your viral stock to obtain a higher titer. You will need to perform the filtration step first before concentrating your viral stock.

Concentrating Viral Particles

You should expect your viral titer to increase at least 60-fold after concentrating. You should also expect a possible 30-50% loss in total viral yield.

1. Pipette supernatant into a sterile SW28 ultracentrifuge tube, centrifuge at 23,000 rpm for 1.5 hours at 4°C.

2. After ultracentrifugation, carefully aspirate and discard the supernatant.

3. Pipette the desired resuspension volume of DMEM (no serum) onto the pellet at the bottom of the tube. The minimum resuspension volume per pellet of supernatant collected from a 100-mm plate is 100µl as it is difficult to resuspend in less volume. For one SW28 tube, which holds about 35ml of supernatant, we recommend resuspending in ~250µl.

4. The visible pellet is made up mostly of serum proteins from the culture media of the transfected cells. The viral particles need to be dislodged from this protein pellet. After adding the DMEM to the pellet, let it sit for 5-10 minutes then gently pipette up and down about 30 times trying to avoid formation of bubbles.

5. Transfer the resuspended pellet to a sterile microfuge tube and centrifuge at full speed for 3-4 minutes. This will pellet the serum proteins, which adhere to the bottom of the tube. After centrifugation, transfer the supernatant to a new microfuge tube and then aliquot into multiple vials.

6. Store aliquots at -80°C.

7. Proceed to Transduction and Titering Procedure.

Long-Term Storage

Place viral stocks at -80°C for long-term storage. Avoid freezing and thawing as it will 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.

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. **Note:** If you have generated a lentiviral stock of the expression control (e.g. pGIPZ™ Non-Silencing), we recommend titering this stock as well.

1. The day before transduction, seed a 24-well tissue culture plate with TLA-HEK293T™ cells at 5 x 10^4 cells per well in DMEM (10% FBS, 1% pen-strep).

   The following day, the well should be no more than 40-50% confluent. TLA-HEK293T (Open Biosystems Catalog no. HCL4517).

2. When ready, make dilutions of the viral stock in a round bottom 96 well plate using Dilution Media (DMEM containing 0.5% FBS and 8µg/ml polybrene). Utilize the plate as shown in Figure 2 using one row for each virus stock to be tested. Use the procedure below (starting at step 4) for dilution of the viral stocks. The goal is to produce a series of 5-fold dilutions to reach a final dilution of 390625-fold.

   Polybrene is a cation that is often pre-incubated with the virus particles to give it a net positive charge, which helps counteract the negatively-charged cell surface membrane. Polybrene - [Sequabrene™, Sigma Catalog no. S-2667]).

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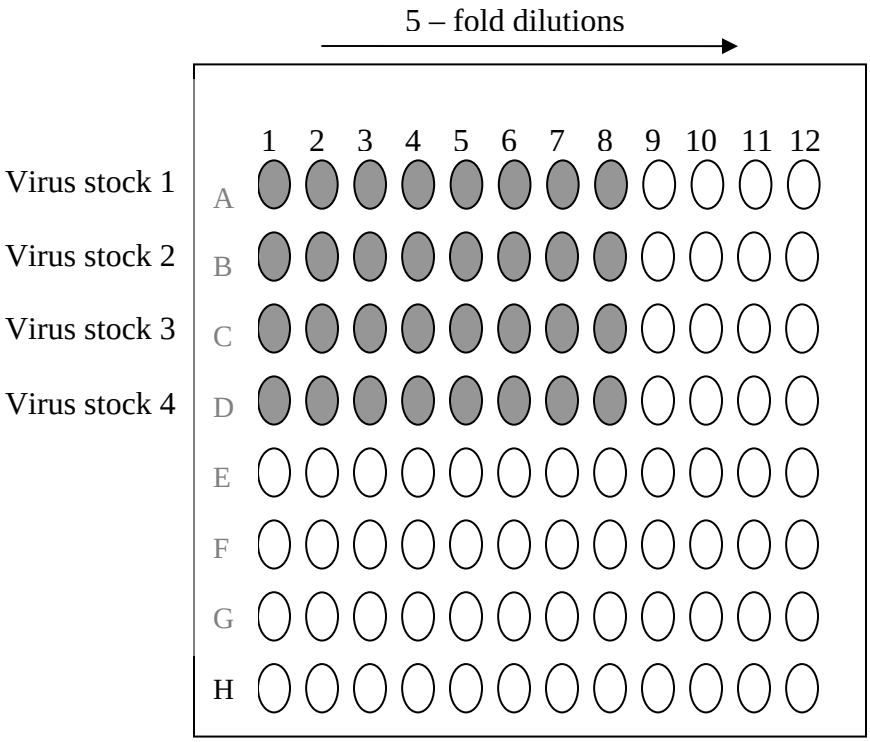

Figure 2. Five-fold serial dilutions of virus stock.

3. To each well add 80µl of Dilution Media.

4. Add 20µl of thawed virus stock to each corresponding well in column 1 (1:5 dilution).

   Pipette contents of well up and down 10-15 times. Discard pipette tip.

5. With new pipette tips, transfer 20µl from each well of column 1 to the corresponding well in column 2.

   Pipette 10-15 times and discard pipette tips.

6. With new pipette tips, transfer 20µl from each well of column 2 to the corresponding well in column 3.

   Pipette 10-15 times and discard pipette tip.

7. Repeat transfers of 20µl from columns 3 through 8, pipetting up and down 10-15 times and changing pipette tips between each dilution.

   It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions. Pre-incubate the dilutions of the virus stock for 5 minutes at room temperature.

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8. Label 24-well plate as shown in Figure 3 using one row for each virus stock to be tested.

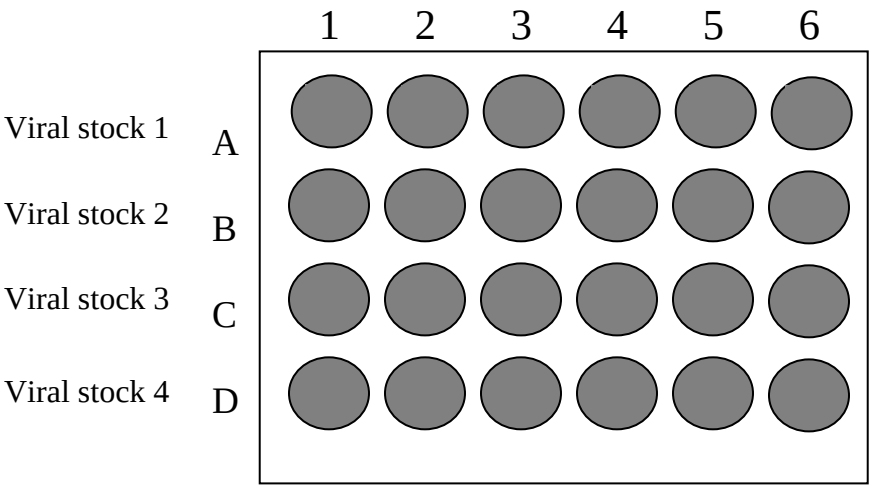

Figure 3. Twenty four well tissue culture plate, seeded with TLA-HEK293T™ cells, used to titer the virus.

9. Remove culture media from the cells in the 24-well plate.

10. Add 150µl of Transduction Media (same as Dilution Media without polybrene) to each well.

11. Transduce cells by adding 25µl of diluted virus from the original 96-well plate (in Figure 2) to a well on the 24-well destination plate (in Figure 3) containing the cells.

For example, transfer 25µl from well A2 of the 96-well plate into well A1 in the 24-well plate.

Well (Row A, B, C, or D)		*Volume diluted virus used*	*Dilution Factor*
Originating (96-well plate)	*Destination (24-well plate)*		
A1		*25µl*	*5 **
A2	*A1*	*25µl*	*25*
A3	*A2*	*25µl*	*125*
A4	*A3*	*25µl*	*625*
A5	*A4*	*25µl*	*3125*
A6	*A5*	*25µl*	*15625*
A7	*A6*	*25µl*	*78125*
A8		*25µl*	*390625 **

*

Please note that when expecting very high or very low titers, it would be advisable to include either well 8 or well 1 respectively.

12. Incubate transduced cultures at 37°C for 4 hours.

13. Remove transduction mix from cultures and add 1ml of DMEM (10% FBS, 1% Pen-Strep).

14. Culture cells for 48 hours.

15. Count the TurboGFP expressing cells or colonies of cells.

Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 4 illustrates this principle of counting.

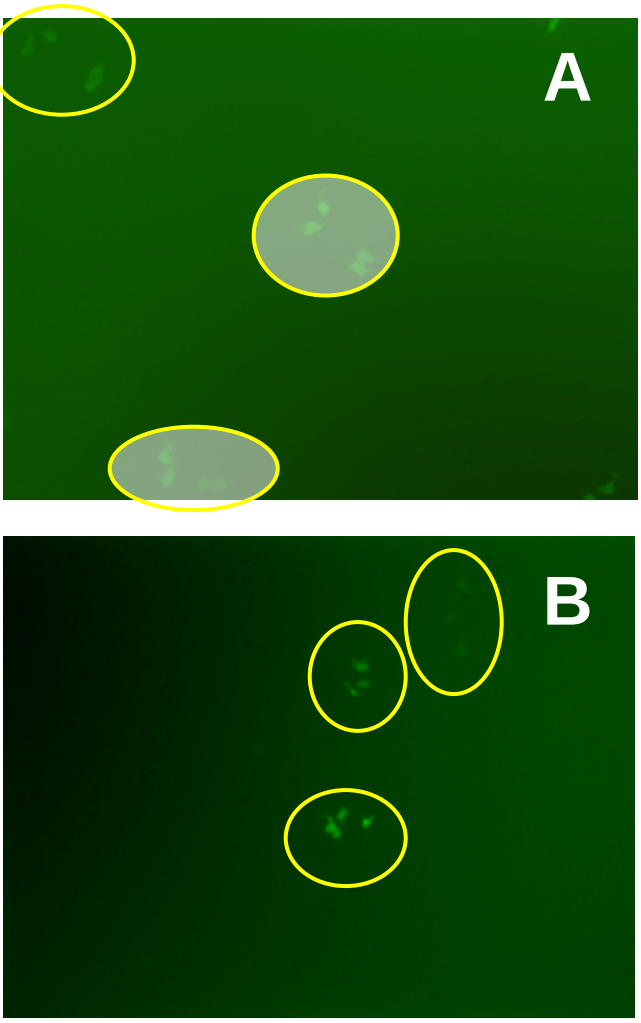

Figure 4. Examples of individual colonies.

16. Transducing units per ml (TU/ml) can be determined using the following formula:
# of TurboGFP positive colonies counted x dilution factor x 40 = #TU/ml

Example: 55 TurboGFP positive colonies counted in well A3.
55 (TurboGFP positive colonies) x 3125 (dilution factor) x 40 = 6.88×10^6 TU/ml

Transduction and Analysis

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral vector into the mammalian cell line of choice and assay for expression of your recombinant protein. Guidelines are provided below.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, you will need to transduce the lentiviral vector into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of transducing units per cell. Although this is cell line dependent, this generally correlates with the number of integration events and as a result, level of expression.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell, its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, after you have tittered it, we recommend using a range of MOIs (e.g. 0, 0.5, 1, 2, 5, 10, 20) to determine the MOI required to obtain optimal expression for your particular application. It should be noted that to achieve single copy knockdown, an MOI of 0.3 is generally used, as less than 4% of your cells will have more than one insert.

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Expression Control

In general, we have found that 60-70% of the cells in an actively dividing cell line express a shRNA when transduced at an MOI of ~1. Some non-dividing cell lines transduce lentiviral constructs less efficiently. For example, only about 50% of the cells in a culture of primary human fibroblasts express a shRNA when transduced at an MOI of ~1. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI to achieve optimal expression levels for your recombinant protein.

If you have generated the positive control lentiviral construct (i.e. pGIPZ™ Non-Silencing), we recommend using the lentiviral stock to help you determine the optimal MOI for your particular cell line and application. Once you have transduced the control lentivirus into your mammalian cell line of choice, the gene encoding the fluorescent protein will be constitutively expressed and can be easily assayed.

Puromycin Kill Curve and Puromycin Selection

Determining Puromycin Dose-Response - In order to generate stable cell lines, it is important to determine the minimum amount of puromycin required to kill non-transfected/transduced cells. This can be done by generating a puromycin kill curve.

Puromycin Kill Curve

1. On day 0 plate 5 - 8 x 10^4 cells per well in a 24-well plate in enough wells to carry out your puromycin dilutions. Incubate overnight.

2. Prepare media specifically for your cells containing a range of antibiotic, for example: 0 - 15µg/ml puromycin.

3. The next day (day 1) replace the growth media with the media containing the dilutions of the antibiotic into the appropriate wells.

4. Incubate at 37°C.

5. Approximately every 2 - 3 days replace with freshly prepared selective media.

6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 1- 4 days under puromycin selection.

7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 1 - 4 days from the start of antibiotic selection (see Figure 5).

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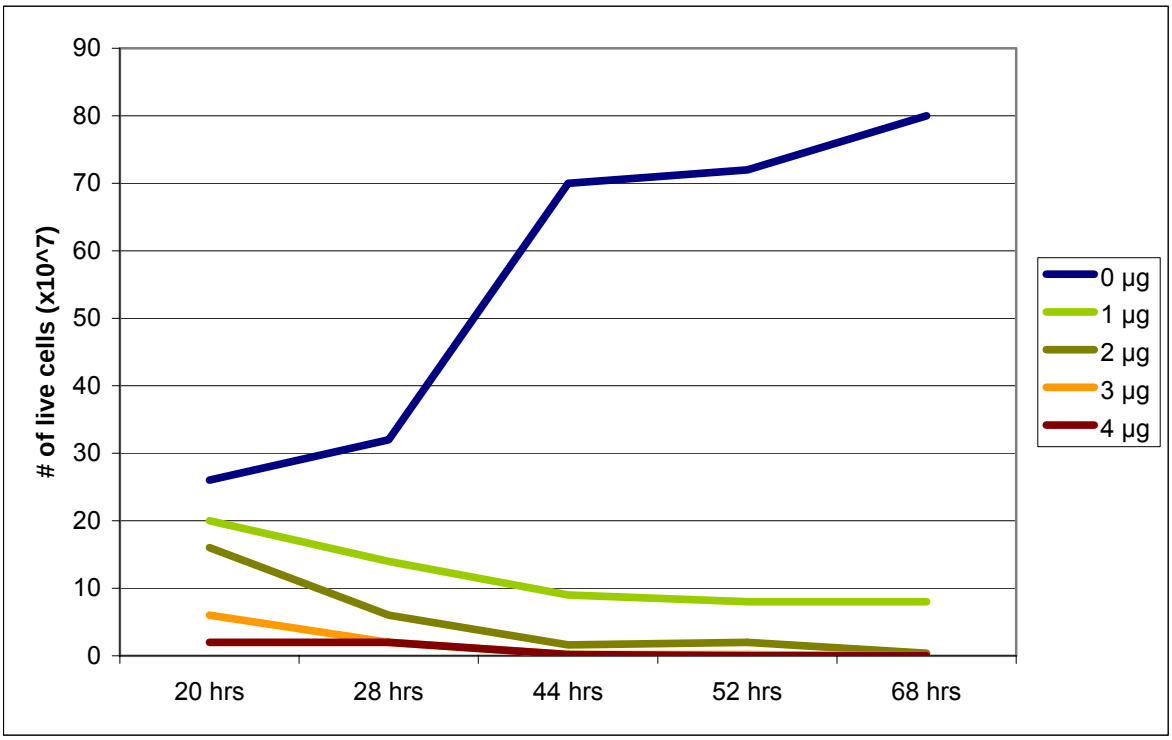

Figure 5. Puromycin kill curve depicting cell death in relation to increasing doses of puromycin over time.

Puromycin Selection of Transduced Cells

1. On day 0 plate 5 - 8 x 10^4 cells per well in a 24-well plate. Incubate overnight.

2. Prepare media specifically for your cells containing the concentration of puromycin you selected based on the above "kill curve".

3. The next day (day 1), remove the medium and add the virus to the MOI you wish to use.

   Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media. If you are using concentrated virus you are likely to use very little virus volume and a lot of serum-free media; if you are using unconcentrated virus you will find you need much more virus volume.

4. Approximately 6-8 hours post-transduction, add an additional 1ml of full media (serum plus pen/strep if you are using it) to your cells and incubate overnight.

5. At 48 hours post transduction, replace the full growth media with full growth media containing the puromycin into the appropriate wells. Incubate.

6. Approximately every 2-3 days replace with freshly prepared selective media.

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7. Monitor the cells daily and observe the percentage of surviving cells as well as the level and total percentage of TurboGFP expression. At some time point almost all of the cells surviving selection will be expressing TurboGFP. Optimum effectiveness should be reached in 3-10 days with puromycin.

   Please note that the higher the MOI you have chosen the more copies of the shRNA and puromycin resistance gene you will have per cell. When selecting on puromycin, it is worth remembering that at higher MOIs, cells containing multiple copies of the resistance gene can withstand higher puromycin concentrations than those at lower MOIs. Adjust the concentration of puromycin to a level that will select for the population of transduced cells you wish to select for, without going below the minimum antibiotic concentration you have established in your "kill curve".

8. Proceed to extract RNA for knock down evaluation by quantitative PCR.

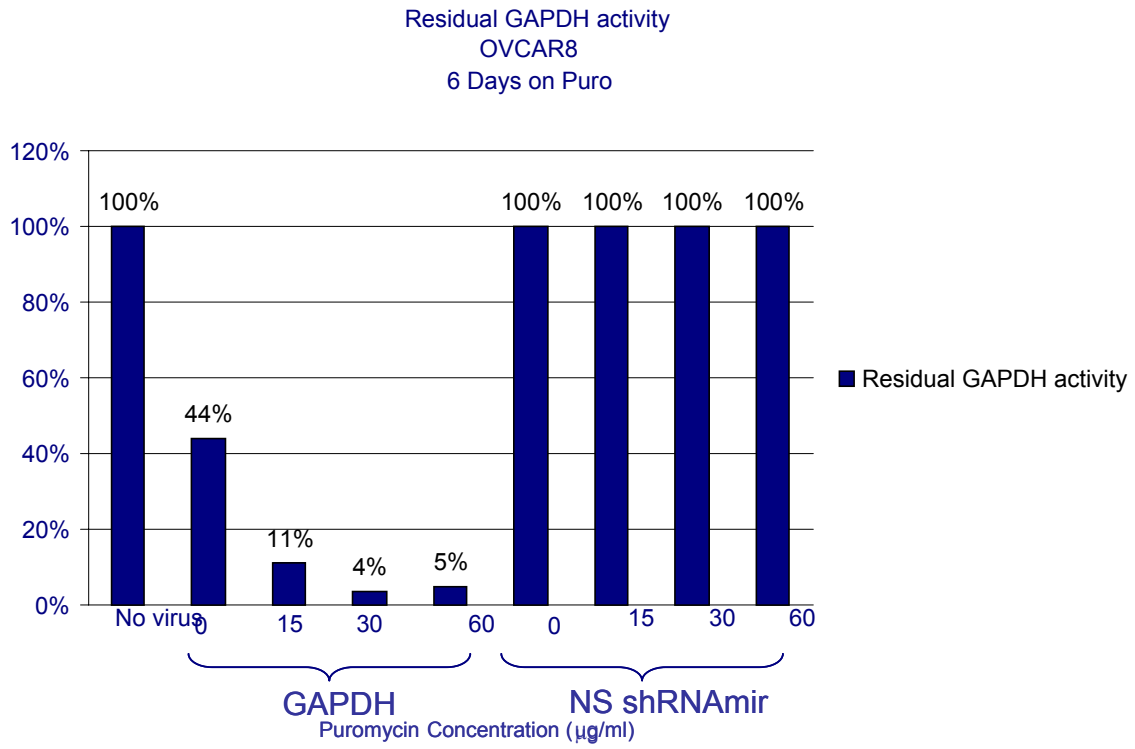

Figure 6. Residual GAPDH activity, as determined by quantitative reverse transciptase PCR, after transduction with a shRNA against GAPDH and puromycin selection at various concentrations.

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General Considerations:

TLA-HEK293T cells are cultured in a complete growth medium at 37ºC with 5% CO_2. TLA-HEK293T cells detach easily from the culture dish surface. Therefore, handle the cells gently when replacing the culture medium or during washing.

Starting Cells from Frozen Cell Stock:

1. Remove the TLA-HEK293T packaging cell line from liquid nitrogen and carry out a 'quick thaw'. Float the cells in the 37ºC water bath for 2-5 minutes until nearly (~80%) thawed.

   TLA-HEK293T stocks are frozen in 80% DMEM media, 10% fetal calf serum, and 10% DMSO, unless otherwise noted. Once cells are thawed, it is important to dilute the cells 1:10 in growth media immediately to reduce the potentially toxic effects of the DMSO preservative on the cells.

2. Remove the cells from the vial and add slowly into a 15ml conical tube containing 10ml pre-warmed media.

3. Centrifuge for 3 minutes ~1000xg to pellet cells and remove the supernatant.

4. Add 14ml of media and transfer cells to a T25 flask or a 100mm culture dish.

   Many protocols recommend removal of freeze medium from the cells following thawing. This is cell line dependent; some cells are more sensitive to the DMSO in the freeze medium. TLA-HEK293T cells do not require the removal of DMSO prior to seeding in the flask.

5. Place the cells in the 37°C incubator with 5% CO_2.

Cell Maintenance

1. Fresh medium should be added to the cells every 3 days or as required by the growth rate of the cells.

2. TLA-HEK293T cells should always be treated very gently as they detach easily from the plate.

3. Add an appropriate volume of pre-warmed complete medium to the cells. You may first need to rinse the cells with PBS or media prior to feeding the cells.

   This is typically done if there is a high degree of dead cells or debris in the culture, as would be expected after a thaw.

4. Return the cells to the 37°C incubator with 5% CO_2.

Sub-culturing/Passaging of Cells

1. TLA-HEK293T cells are passaged when they are 90% confluent to a ratio of 1:15 to 1:20 for general maintenance.

   Cells can be passaged using a smaller ratio but will then reach confluency quicker and will need to be passaged more frequently (for example 1:5).

2. Carefully aspirate the growth media from the cells. This is best done by tilting the flask or plate and removing the medium without touching the cell surface.

3. Gently wash cells with PBS.
4. Trypsinize the cells (see Table 5).

   Place plate in the 37ºC incubator for ~2 minutes for cells to release from the plate.

5. Add complete cell growth media to resuspend cells and inactivate the trypsin.
6. Pipette cells up and down ~5 times with a 10ml strip pipette to get a single cell suspension, while avoiding frothing of media.
7. Plate cells into new sterile flasks or plates containing complete growth medium (see Table 6). Place the cells at 37°C with 5% CO_2.

Table 5. Trypsinization and resuspension volumes for routinely used vessels.

Cell Culture Vessel	PBS Wash (ml)	Trypsin (ml)	Resuspension Cell Growth Media (ml)	Recommended Volume Media in New Flask (ml)
T-25 or 100mm	2.5	1	5-10	5-10
T-150	10	2	10	30-40
T-175	10	2	10	35-50

Table 6. Flask and plate surface areas and recommended volumes.

Flask Type	Growth Area Per Well (cm^2)	Volume Growth Media Per Well
T-175	175	35-50
T-150	150	30-40
100 mm dish	55	10
T-25	25	10
6 well	9.5	3
12 well	4	2
24 well	2	1
48 well	1	0.5
96 well	0.32	0.1
8 well chamber	0.8	0.4

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1L of Media for TLA-HEK293T™
885ml DMEM High Glucose, w/o sodium pyruvate
100ml FBS
10ml Pen/Strep (stock 10,000 I.U. Penicillin and 10,000mg/ml streptomycin)
5ml L-Glutamine (stock 200mM L-Glutamine)

Freeze Medium recipe:
90% regular media with serum
10% DMSO

Final Concentrations and Vendors for Medium Components:
DMEM High Glucose, w/o sodium pyruvate (Mediatech MT10017CV)
10% FBS
1mM glutamine additional (Hyclone SH30034.02, 200mM)
1x Pen/Strep (Mediatech MT30002CI)

Other reagents:
PBS without Ca_{2+}/Mg_{2+} (Mediatech 21-031-CM)
Trypsin/EDTA (Mediatech 25-053-CI)
DMSO (Calbiochem 317275)

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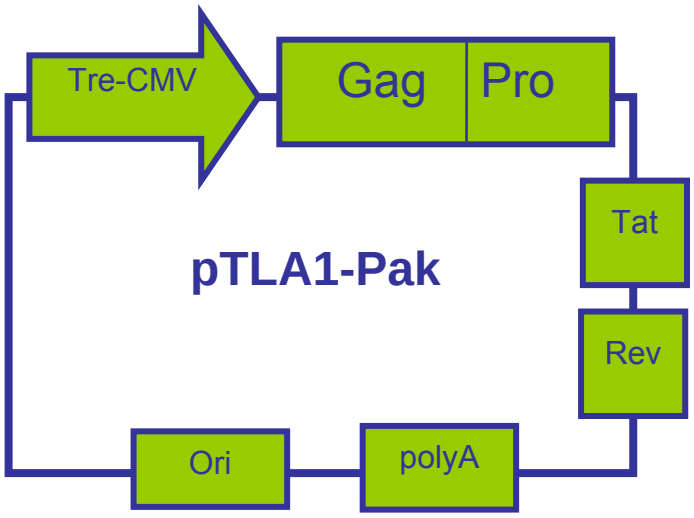

Human cytomegalovirus (Tre-CMV)	RNA polymerase II promoter that permits high-level expression of the lentiviral *gag* and *pro* genes in mammalian cells.
gag	Virion structural proteins for forming the virion core and ribonucleoprotein complex within the core.
pro	Aspartyl-protease that cleaves the Gag, Gag-Pro, Gag-Pro-Pol polyproteins to produce viral proteins in their mature forms.
rev	Viral protein that binds to the RRE and facilitates the transport of unspliced and incompletely spliced mRNAs to the cytoplasm.
tat	Transcriptional activator of lentiviral gene expression, enhancing LTR-directed transcription.
polyA	Allows transcription termination and polyadenylation of the mRNA.
Ori	Permits high-copy replication and maintenance in *E. coli.*

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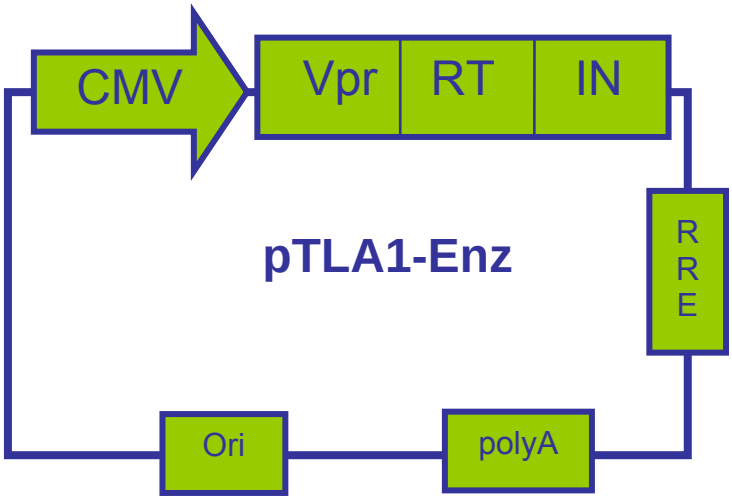

Human cytomegalovirus (CMV)	RNA polymerase II promoter that permits high-level expression of the lentiviral *RT* and *IN* genes in mammalian cells.
Vpr	Viral protein that shuttles the RT and IN into the viral particle.
RT	Reverse Transcriptase; DNA polymerase that copies RNA into DNA.
IN	Integrase; Enzyme responsible for inserting the linear double-stranded DNA copy of the lentiviral genome into host cell DNA.
RRE	Binding site for the Rev protein that aids in the transport of unspliced and singly-spliced RNAs from the nucleus to cytoplasm.
polyA	Allows transcription termination and polyadenylation of the mRNA.
Ori	Permits high-copy replication and maintenance in *E. coli.*

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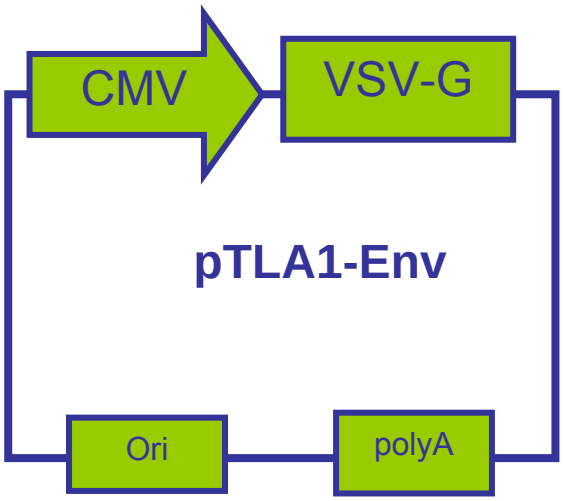

Human cytomegalovirus (CMV)	RNA polymerase II promoter that permits high-level expression of the lentiviral VSV-G gene in mammalian cells.
VSV-G	Envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped lentivirus.
polyA	Allows transcription termination and polyadenylation of the mRNA.
Ori	Permits high-copy replication and maintenance in *E. coli.*

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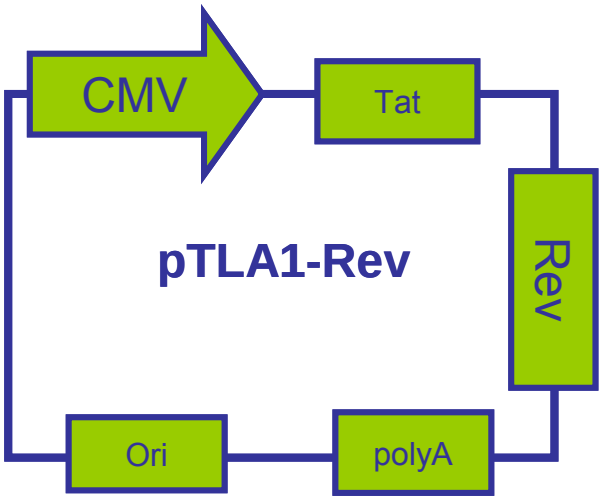

Human cytomegalovirus (CMV)	RNA polymerase II promoter that permits high-level expression of the lentiviral *tat* and *rev* genes in mammalian cells.
tat	Transcriptional activator of lentiviral gene expression, enhancing LTR-directed transcription.
rev	Viral protein that binds to the RRE and facilitates the transport of unspliced and incompletely spliced mRNAs to the cytoplasm.
polyA	Allows transcription termination and polyadenylation of the mRNA.
Ori	Permits high-copy replication and maintenance in *E. coli.*

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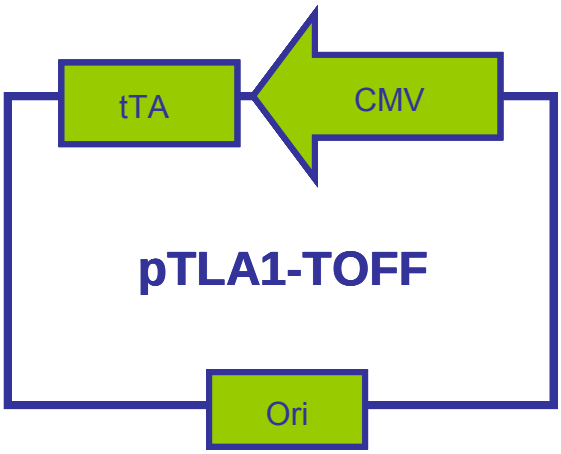

Human cytomegalovirus (CMV)	RNA polymerase II promoter.
tTA	Transactivator protein that binds to the TRE in the absence of doxycyline and allows transcription through the minimal CMV promoter.
Ori	Permits high-copy replication and maintenance in *E. coli.*

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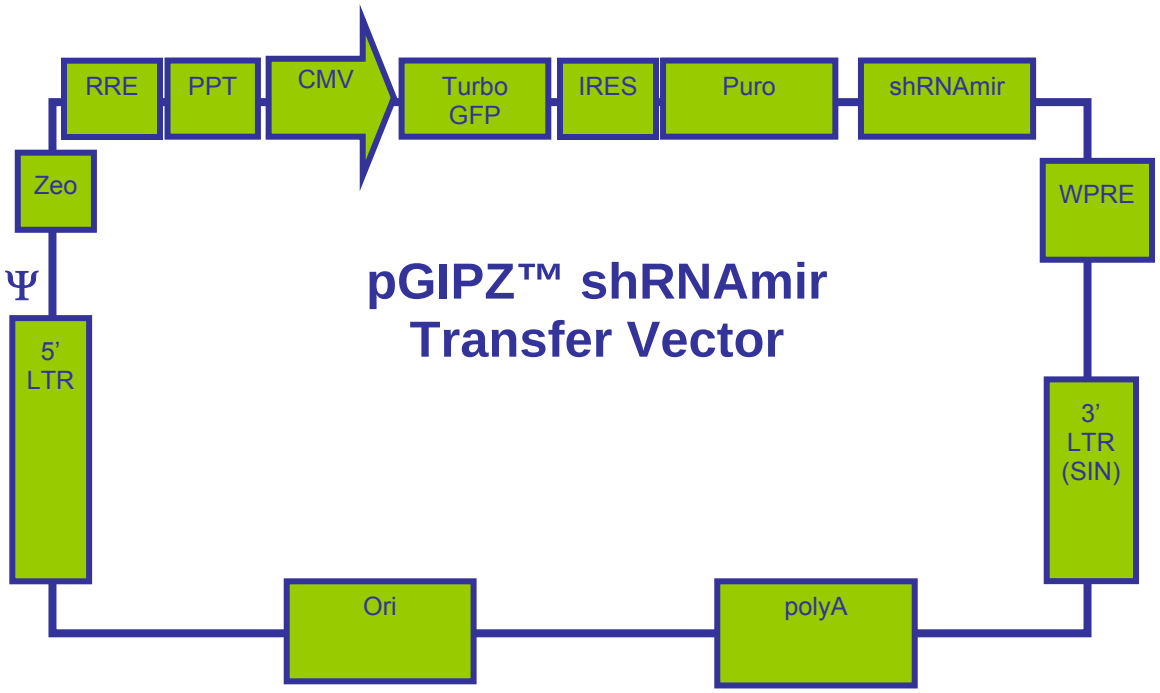

CMV	RNA polymerase II promoter that permits high-level expression of the lentiviral gag and pro genes in mammalian cells.
Ψ (Psi)	Region of viral RNA responsible for directing packaging.
PPT	Purine-rich sequence cleaved during reverse transcription to produce the RNA primer for synthesis of viral DNA.
wpre	Post-transcriptional regulatory element derived from the woodchuck hepatitis virus to increase vector independent expression levels of the transgene.
3' & 5' LTR	Long terminal repeat region composed of U3-R-U5 (5' to 3').
SIN	Deletion of the transcriptional enhancers & promoter in the U3 region of the 3' LTR.
RRE	Binding site for the Rev protein that aids in the transport of unspliced RNAs from the nucleus to cytoplasm.
TurboGFP	Green fluorescent protein utilized to track shRNAmir expression.
Puro	Puromycin-N-acetyl transferase, mammalian drug selectable marker.
shRNAmir	microRNA-30 flanking a stem-loop-stem structure.
polyA	Allows transcription termination and polyadenylation of the mRNA.
Ori	Permits high-copy replication and maintenance in *E. coli.*
Amp	Allows selection of the plasmid in *E. coli.*