Technical Manual

Thermo Scientific Open Biosystems LentiORF pLEX-MCS Vector

Catalog #: OHS4735

Product Description

The pLEX-MCS Empty Vector is the lentiviral pLEX vector containing a multiple cloning site. The pLEX vector backbone is designed for transient or stable transfection and for lentiviral production and subsequent transduction in a target cell line resulting in the over expression of your gene of choice.

The pLEX-MCS Empty Vector is provided as a bacterial culture of PrimePlus *E. coli* in LB media with 8% glycerol, 25 µg/ml zeocin and 100 µg/ml carbenicillin.

Shipping and Storage

The pLEX-MCS Empty Vector is shipped on wet ice and should be stored at –80°C upon receipt. All cultures are checked for growth prior to shipment.

Vector Information

Features of the LentiORF pLEX-MCS Lentiviral Vector (Figures 1 and 2, Tables 1 and 2) that make it a versatile tool for over expression studies include:

- Ability to perform transfections or transductions using the replication incompetent lentivirus
- Amenable to *in vitro* and *in vivo* applications
- Puromycin drug resistance marker for selecting stable cell lines
- Multiple cloning site that allows for the cloning of any ORF into a lentiviral expression vector

Table 1. Features of the LentiORF pLEX-MCS Vector

Vector Element	Utility
5'LTR (wt)	5' long terminal repeat
ZeoR	Bacterial selection marker
CMV Promoter	RNA polymerase II promoter
IRES	Internal ribosome entry site
PuroR	Mammalian selectable marker for selection after viral transduction
WPRE	Woodchuck hepatitis post-transcriptional regulatory element
pUC ori	High copy replication and maintenance of plasmid in *E.coli*
SIN-LTR	3' self inactivating long terminal repeat
AmpR	Bacterial selectable marker

Antibiotic Resistance

The LentiORF pLEX-MCS contains 3 antibiotic resistance markers (Table 2).

Table 2. Antibiotic Resistances Conveyed by the LentiORF pLEX-MCS Vector

Antibiotic	Concentration	Utility
Zeocin	100 µg/ml	Bacterial selection marker
Ampicillin (carbenicillin)	100 µg/ml	Bacterial selection marker
Puromycin	Variable	Mammalian selection marker

Sequencing Primers

Table 3. Primer information

Primer Name	Length	Tm	% GC	Sequence 5'-3'
pLEX-MCS-FWD	20 bp	59.8 °C	45	CACCAAAATCAACGGGACTT
pLEX-MCS-REV	24 bp	60.3 °C	50	ATATAGACAAACGCACACCGGCCT

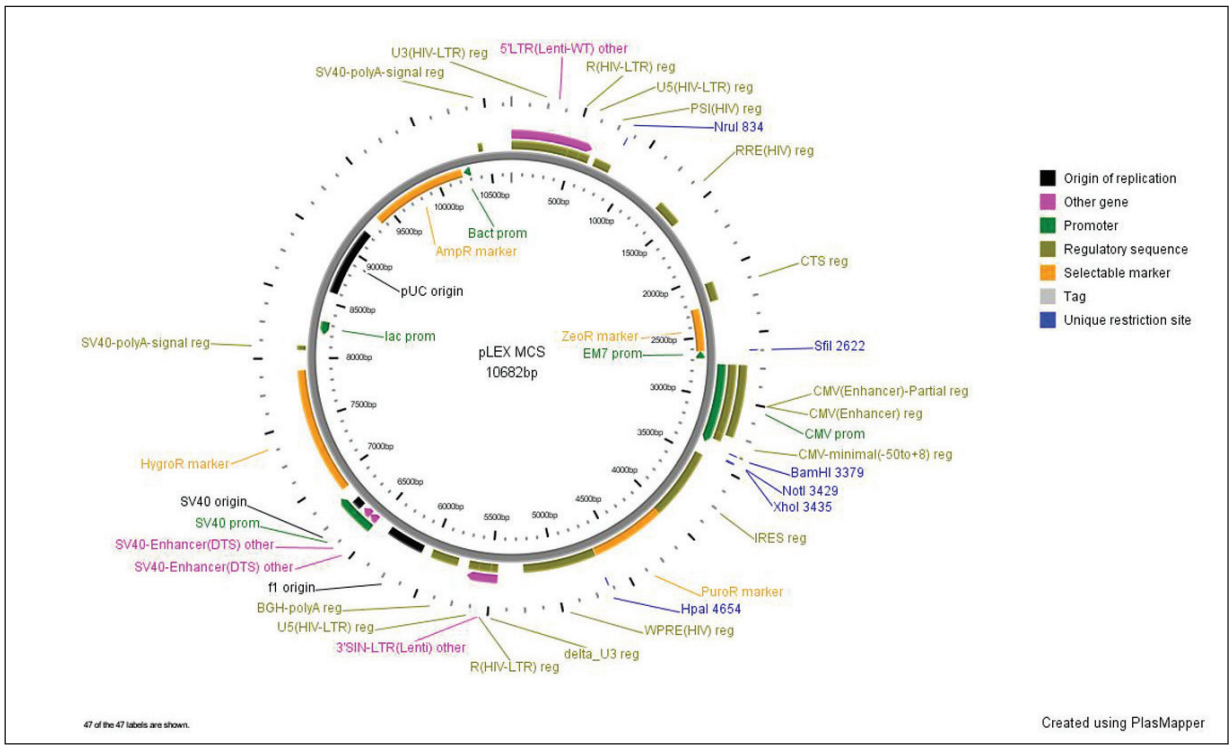

Figure 1. Map of LentiORF pLEX-MCS Vector

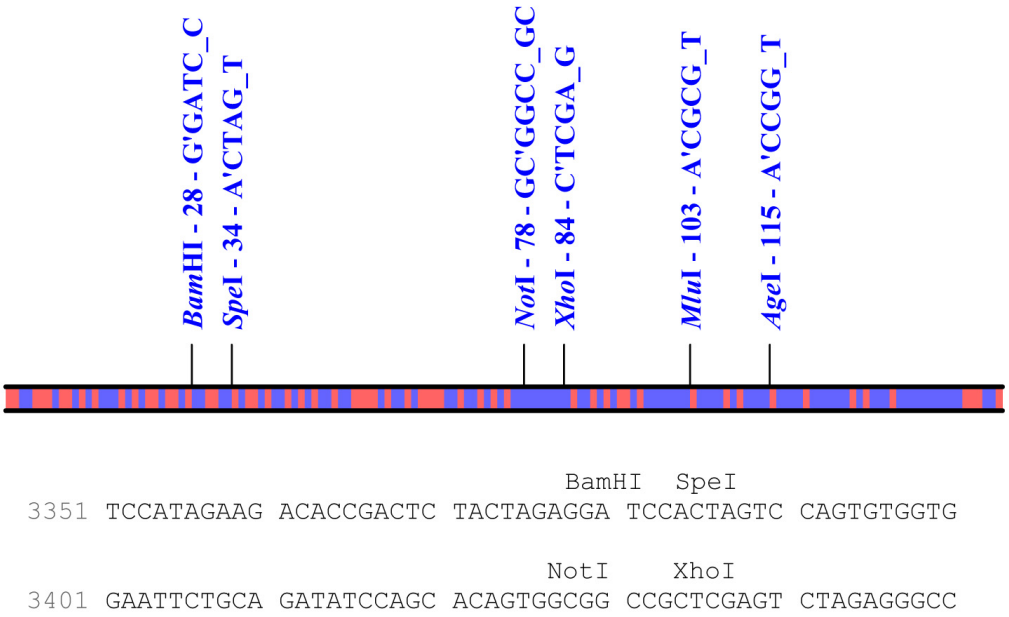

Figure 2. Multiple cloning site of LentiORF pLEX-MCS Vector

Protocol I - Replication

Culture conditions for replication of shRNA constructs

For archive replication, grow all pLEX clones at 30°C in LB-Lennox (low salt) media plus 25 µg/ml zeocin and 100 µg/ml carbenicillin in order to provide maximum stability of the clones.

Prepare media with glycerol and the appropriate antibiotics. Use the minimal volume of inoculant (0.5-1 µl in 1 ml LB). Incubate with shaking at 30°C for 16-20 hours. Freeze at -80°C for long term storage. Avoid long periods of storage at room temperature or higher in order to control background recombination products.

2XLB broth* (low-salt) media preparation

Peptone	20 g/l
Yeast Extract	10 g/l
Appropriate antibiotic(s) at recommended concentration(s)	
**Glycerol	8% for long term storage

**LB media may be substituted.*

***Glycerol can be omitted from the media if you are culturing for plasmid preparation. If making copies of the constructs for long term storage at -80°C, 8% glycerol is required.*

Protocol II - Plasmid Prep

Culture conditions for individual plasmid preparations

Most plasmid mini-prep kits recommend a culture volume of 1-10 ml for good yield. For lentiORF constructs, 5 ml of culture can be used for one plasmid mini-prep generally producing 5-10 µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the LentiORF of interest, store at –80°C until ready to begin.
2. To prepare plasmid DNA, thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Take 10 µl inoculum from the glycerol stock into 3-5 ml of LB (low salt) with 100 µg/ml carbenicillin and 25 µg/ml zeocin. Incubate at 37°C for 16 hours with vigorous shaking. Return the glycerol stock(s) to -80°C. If a larger culture volume is desired, use the 3-5 ml overnight culture as a starter inoculum. Incubate at 37°C for 16 hours with vigorous shaking.
4. Pellet the 3-5 ml culture and begin preparation of plasmid DNA.
5. Run 3-5 µl of the plasmid DNA on a 1% agarose gel. pLEX vector without an ORF is approximately 10.7 kb.

Note: Due to the tendency of all viral vectors to recombine, we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock or the colony glycerol stock for each plasmid preparation.

Protocol III - Transfection

The protocol below is optimized for transfection of the plasmid DNA into HEK293T cells in a 24-well plate using serum free media. If a different culture dish is used, adjust the number of cells, volumes and reagent quantities in proportion to the change in surface area (Table 3).

It is preferable that transfections be carried out in medium that is serum-free and antibiotic-free. A reduction in transfection efficiency occurs in the presence of serum, however it is possible to carry out successful transfections with serum present (see Transfection Optimization).

Warm Thermo Scientific Open Biosystems Express-In to ambient temperature (approximately 20 minutes) prior to use. Always mix well by vortex or inversion prior to use.

Maintain sterile working conditions with the DNA and Open Biosystems Express-In™ mixtures as they will be added to the cells.

1. The day before transfection, plate the cells at a density of 5 x 10^4 cells per well of a 24-well plate.

Table 3. Suggested amounts of DNA, medium and Express-In reagent for transfection of plasmid DNA into adherent cells.

Tissue Culture Dish	Surface Area per Plate or Well (cm^2)	Total Serum-Free Media Volume per Well (ml)	Plasmid DNA (µg)*	Express-In (µg)**
60 mm	20.0	2.0	4.0	21.0
35 mm	8.0	1.0	2.0	10.0
6-well	9.4	1.0	2.0	10.0
12-well	3.8	0.5	1.0	5.0
24-well	1.9	0.25	0.5	2.5
96-well	0.3	0.1	0.1 - 0.2	0.5 - 1.0

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

**Recommended starting amount of Express-In reagent. See Transfection Optimization.

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

2. On the day of transfection, form the DNA/Express-In transfection complexes.
   The principle is to prepare the plasmid 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 Express-In) will be added to each other and incubated for 20 minutes prior to addition to the cells. This enables the DNA/Express-In complexes to form.
   a. For each well to be transfected, dilute 500 ng shRNA plasmid DNA into 50 µl (total volume) of serum free medium in a microfuge tube.
   b. For each well to be transfected, dilute 2.5 µg (2.5 µl) of Express-In into 50 µl (total volume) serum free medium into a separate microfuge tube.
   c. Add the diluted DNA (step a) to the diluted Express-In reagent (step b), mix rapidly then incubate for 20 minutes at room temperature.
      This will give a 1:5 DNA: Express-In ratio which is recommended for optimal transfection into HEK293T cells. Your total volume will be 100 µl at this stage.
   d. Set up all desired experiments and controls in a similar fashion as outlined in Table 4. It is also advisable to set up an Express-In only control.

Table 4. Quantities of DNA for transfection experiments

Type of Transfection Experiment	shRNA Plasmid DNA (ng)	Reporter* (ng)	Carrier DNA** (ng)	Serum-free Medium (final volume in µl)
Plasmid DNA	500 – ORF	0	0	50
Transfection efficiency	0	500	0	50

3. Aspirate the growth medium from the cells. Add an additional 150 µl of serum free medium to each of the tubes containing transfection complexes and mix gently. Add the 250 µl DNA/Express-In complex mixture to the cells and incubate for 3-6 hours in a CO_2 incubator at 37°C.
   Your total volume will be 250 µl at this stage.

4. Following the 3-6 hour incubation, add an equal volume of growth medium (250 µl) containing twice the amount of normal serum to the cells (i.e. to bring the overall concentration of serum to what is typical for your cell line). Alternatively, the transfection medium can be aspirated and replaced with the standard culture medium (see Note). Return the cells to the CO_2 incubator at 37°C.
   Note: Express-In has displayed low toxicity in the cell lines tested, therefore removal of transfection reagent is not required for many cell lines. In our experience, higher transfection efficiencies have been achieved if the transfection medium is ***not*** *removed. However, if toxicity is a problem, aspirate the transfection mixture after 3-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.*

5. After 48-96 hours of incubation, examine the cells microscopically for the presence of reporter expression where applicable as this will be your first indication as to the efficiency of your transfection. Then assay cells for gene expression or reporter activity by quantitative/real-time QPCR, western blot or other appropriate functional assay; compare to untreated, reporter alone, and negative control.
   Optimal length of incubation from the start of transfection to analysis is dependent on cell type, gene of interest, and the stability of the mRNA and/or protein being analyzed. Quantitative/real-time QPCR generally gives the best indication of expression. The use of western blots to determine expression is very dependent on quantity and quality of the protein, its half-life and the sensitivity of the antibody and detection systems used.

6. If selecting for stably transfected cells transfer the cells to medium containing puromycin for selection. It is important to wait at least 48 hours before beginning selection. Optional-transduction is considered a superior method for generating a stable cell line (Protocol V).
   The working concentration of puromycin needed varies between cell lines. We recommend you determine the optimal concentration of puromycin required to kill your host cell line prior to selection for stable ORF transfectants. Typically, the working concentration ranges from 1-10 µg/ml. You should use the lowest concentration that kills 100% of the cells in 3-5 days from the start of puromycin selection.

Cells Grown In Suspension

Transfection of cells in suspension would follow all the above principles and the protocol would largely remain the same, except that the DNA/Express-In mixture should be added to cells (post 20 minute incubation for complex formation) to a total volume of 250 µl serum-free medium or to a total volume of 250 µl of medium with serum (no antibiotics).

Transfection Optimization using Express-In

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells. The most important parameters for optimization are DNA to transfection reagent ratio, DNA concentrations and cell confluency. We recommend that you initially begin with the Express-In and DNA amount indicated in Table 4 and 5 and extrapolate the number of cells needed for your vessel size from the number of cells used in a well of a 24-well plate as listed in step 1 of the protocol for delivery of plasmid DNA.

Protocol IV - Packaging Lentivirus

For packaging our lentiviral ORF constructs, we recommend the Thermo Scientific Open Biosystems TransLenti Viral ORF Packaging System (TLP4616, TLP4617). The Open Biosystems TransLenti™ Viral ORF Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus which can be used to deliver and express your gene of interest in either dividing or non-dividing mammalian cells. The TransLenti Viral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes, *et al.* (2001). For protocols and information on packaging lentiORFs with our TransLenti Viral ORF Packaging System, please visit our website (www.thermo.com/openbiosystems).

Protocol V - Transduction

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. This protocol uses the TLA-HEK293T (Thermo Scientific Open Biosystems Catalog # HCL4517) cell line that is available as part of our TransLenti Viral ORF Packaging System. You can use a standard HEK293T cell line as an alternative.

Note: If you have generated a lentiviral stock of the expression control (e.g. pLEX-MCS control vector or pLEX-Jred™-TurboGFP control vector), 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.
2. Make dilutions of the viral stock in a round bottom 96-well plate using Dilution Media (DMEM containing 0.5% FBS and 8 µg/ml polybrene). Utilize the plate as shown in Figure 3 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.
3. Add 80 µl of Dilution Media to each well.
4. Add 20 µl of thawed virus stock to each corresponding well in column 1 (5-fold dilution).
   Pipette contents of well up and down 10-15 times. Discard pipette tip.

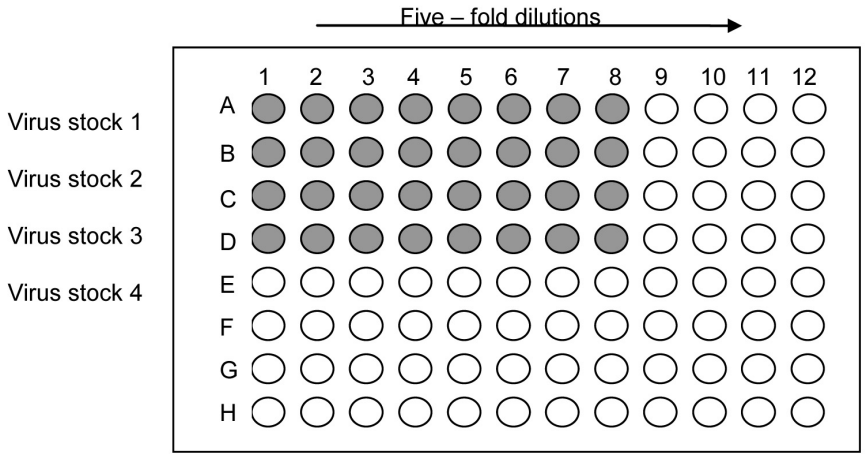

Figure 3. Five-fold serial dilutions of virus stock.

5. With new pipette tips, transfer 20 µl 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 20 µl 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 20 µl 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.
8. Label 24-well plate as shown in Figure 4 using one row for each virus stock to be tested.
9. Remove culture media from the cells in the 24-well plate.
10. Add 150 µl of Transduction Media (same as Dilution Media without polybrene) to each well.
11. Transduce cells by adding 25 µl of diluted virus from the original 96-well plate (Figure 3) to a well on the 24-well destination plate (Figure 4) containing the cells.
    For example, transfer 25 µl from well A2 of the 96-well plate into well A1 in the 24-well plate (Table 5).
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.

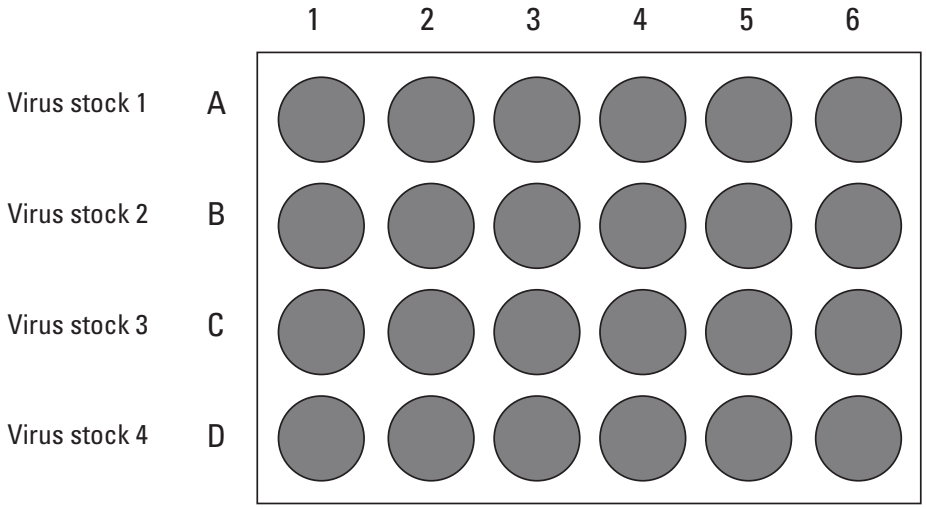

Figure 4. 24-well tissue culture plate, seeded with TLA-HEK293T cells, used to titer the virus.

Table 5. Example of set up for dilutions

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

15. Calculate titer using puromycin selection (see Puromycin Kill Curve and Puromycin Selection below).
    Viral titer is calculated by the number of colonies that are present at the highest dilution (where colonies are present) multiplied by the dilution factor. For example if there were 5 colonies visible in the 105 dilution the titer would be 5 x 10^5 transducing units (TU)/ml.

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.

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 (actively-dividing versus non-dividing), 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 titered 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.

Expression Control

We recommend using the Thermo Scientific Open Biosystems Lentiviral pLEX-Jred-TurboGFP Control Vector 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

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 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 - 15 µg/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.

Puromycin Selection of Transduced Cells

1. On day 0, plate 5 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 4-6 hours post-transduction, add an additional 1 ml 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.
7. Monitor the cells daily and observe the percentage of surviving cells. 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 ORF and puromycin resistance gene you will have per cell. When selecting on puromycin, remember 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 expression evaluation by quantitative QPCR.

References

Table 6. Related Thermo Scientfic Open Biosystems Reagents

Reagent	Catalog #
HEK-293T cell line	HCL4517
Human LentiORF (HA/Myc) Clone	OHS4492
Human LentiORF (Jred)* Clone	OHS4493
Human LentiORF (HA/Myc) Collection	OHS4495
Human LentiORF (Jred) Collection	OHS4496
LentiORF (HA/Myc) Druggable Set	OHS4498
LentiORF (Jred) Druggable Set	OHS4499
pLEX MCS Vector	OHS4735
pLEX Jred TurboGFP Control Vector	OHS4736
Express-In Transfection Reagent 0.5 ml-10 mls	ETR4620-4623
TransLenti Viral ORF Packaging System	TLP4616
TransLenti Viral ORF Packaging System (contains cell line)	TLP4617

*Jred is a trademark of Evrogen.

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Rual JR *et al* (2004). Human ORFeome Version 1.1: a platform for reverse proteomics. Genome Research 14, 2128–2135.

Wilson IA *et al* (1984). The structure of an antigenic determinant in a protein. Cell 37, 776–778.

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