TECHNICAL MANUAL

Precision LentiORF™ Collection

Cat #OHS5832, 5833, 5834, 5898, 5900

Important safety note

Please follow the safety guidelines for use and production of vector-based lentivirus as set by your institution's biosafety committee.

- For glycerol stocks of *E. coli* containing lentiviral plasmids, BSL1 guidelines should be followed
- For handling and use of lentiviral products to produce lentiviral particles, BSL2 or BSL2+ guidelines should be followed
- For handling and use of lentiviral particle products, BSL2 or BSL2+ guidelines should be followed

Please note that Precision LentiORF vectors are not compatible with third generation packaging systems, due to the requirement of the expression of tat, which third generation systems do not contain. We recommend the Dharmacon™ Trans-Lentiviral™ Packaging System for use with our vectors.

**Our Precision LentiORFs are available in glycerol stock or viral particle format. If viral particle format is purchased, begin work with Protocol IX – Determining Relative Transduction Efficiency on page 11.

Product description

The Precision LentiORF collection is a library of expression-ready lentiviral open reading frames (ORFs) generated using gene content from the fully sequenced ORFeome Collaboration Collection. Its unique design expresses the ORF, the fluorescent reporter and the selection marker from one promoter, providing a visual indicator for ORF expression and a mechanism for selecting transduced cells (Figure 1 and Table 1). When used in lentiviral format, precise control of expression level can be achieved by varying the multiplicity of infection (MOI). This collection provides a robust ORF expression tool for gene analysis. Highlights include:

- Versatile vector design with convenient cloning site for the addition of fusion tags (Figure 2)
- Transfection or transduction options for ORF expression
- Dual marker cassette to optimize transduction and maximize expression of your ORF in the cell population
  - Nuclear localized TurboGFP™ (Evrogen™, Moscow, Russia) for easy tracking
  - Blasticidin S resistance for selection
- Lentiviral transduction extends gene expression to a broad range of cell types
- Fully sequenced ORFs for increased confidence in gene expression results

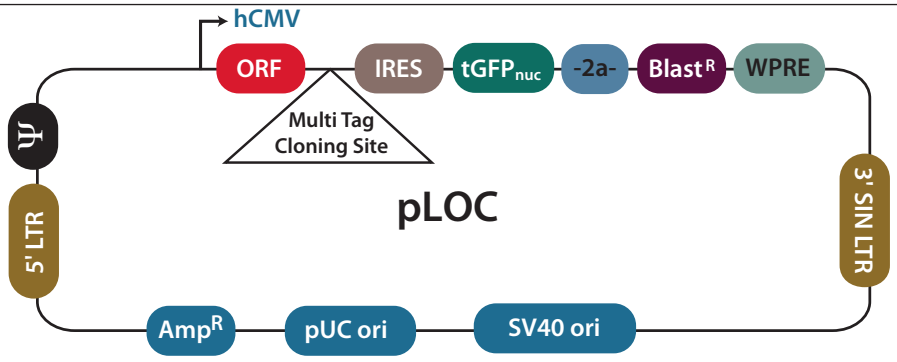

Figure 1. pLOC lentiviral ORF clone.

Table 1. Features of the pLOC vector.

Vector element	Utility
hCMV	Human cytomegalovirus promoter drives strong transgene expression
ORF	Full-length human ORFs from the ORFeome Collaboration Collection
Multi Tag Cloning site	Convenient cloning site for the addition of a purification or tracking tag
IRES	Internal ribosomal entry site allows expression of Evrogen™ TurboGFP™ and Blasticidin S resistance genes in a single transcript
$tGFP_{nuc}$	TurboGFP reporter expressed in the cell nucleus to facilitate visual tracking of transduction and expression
$Blast^R$	Blasticidin S resistance permits antibiotic-selective pressure and propagation of stable integrants
2a	2a self-cleaving peptide allows simultaneous expression of $tGFP_{nuc}$ and Blasticidin S resistance proteins from a single transcript
5' LTR	5' long terminal repeat
3' SIN LTR	3' self-inactivating long terminal repeat for increased lentivirus safety
Ψ	Psi packaging sequence allows viral genome packaging using lentiviral packaging systems
WPRE	Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells

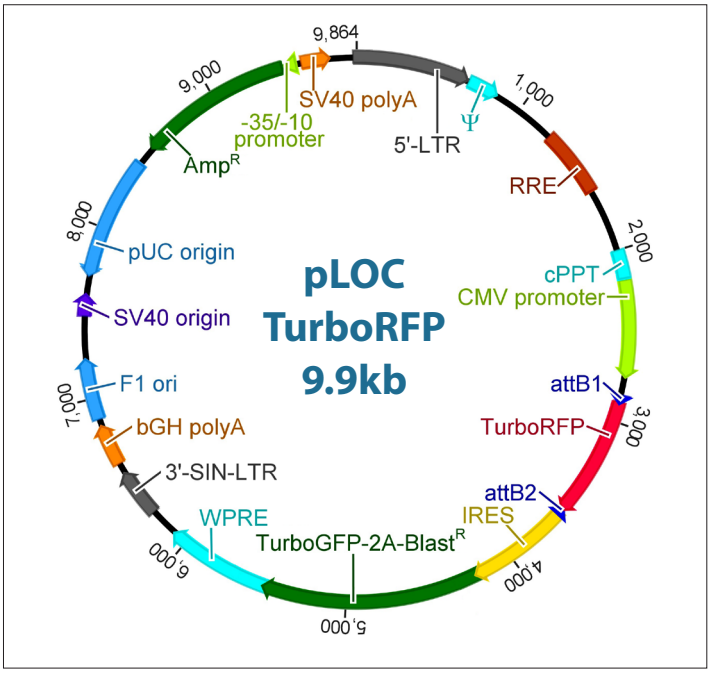

Figure 2. Detailed vector map of pLOC lentiviral vector containing the Evrogen TurboRFP ORF. The lentiviral ORF clone contains attB1 and attB2; the ORF clone contains attL1 and attL2.

The specific sequence of the att site may vary between clones.

Antibiotic resistance

pLOC contains two resistance markers (Table 2).

Antibiotic	Concentration	Utility	Fisher Scientific Cat #
Ampicillin (carbenicillin)	100 µg/mL	Bacterial selection marker (outside LTRs)	BP2648-250
Blasticidin S	variable	Mammalian selectable marker	BP2647-25

Quality control

Each ORF has been end-sequenced to verify identity following the cloning process. The att site, MultiTag cloning site and secondary stop codons have also been sequence verified.

Additional safety information

Historically, the greatest safety risk associated with a lentiviral delivery platform stems from the potential generation of recombinant viruses that are capable of autonomous replication. Our Precision LentiORF platform minimizes these hazards to the greatest degree by combining a disabled viral genome with the proprietary Dharmacon Trans-Lentiviral packaging process. Starting with the HXB2 clone of HIV-1 (GenBank Accession Number K03455), the lentiviral backbone has been modified to eliminate all but essential genetic elements necessary for packaging and integration (including 5' LTR, Psi sequences, polypurine tracts, Rev responsive elements and 3' LTR). The resultant self-inactivating (SIN) vector greatly reduces the probability of producing recombinant particles and limits cellular toxicity often associated with expression of HIV genes.

Additional safety features are incorporated by the manufacturing process itself. Generation of Precision LentiORF lentiviral particles requires a packaging step during which the expression construct containing the ORF sequence is enclosed in a viral capsid. Gene functions that facilitate this process (such as encoded by the structural genes *gag, pol, env*) are distributed amongst multiple helper plasmids which do not contain significant regions of homology. This tactic further minimizes the probability of recombination events that might otherwise generate viruses capable of autonomous replication. Among commercially available lentiviral vector systems, the Trans-Lentiviral Packaging System offers a superior safety profile as the packaging components are separated onto five plasmids. Additionally, expression of gag-pro and tat-rev are under the control of the conditional tetracycline-responsive promoter element (TRE), limiting expression of these viral components strictly to the packaging cell line. A detailed description of the Trans-Lentiviral Packaging System can be found in Wu *et al.* (1).

With these safety measures in place, Precision LentiORF lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities. Any investigator who purchases viral vector products is responsible for consulting with their institution's health and biosafety group for specific guidelines on the handling of lentiviral vector particles. Further, each investigator is fully responsible for obtaining the required permissions for the acceptance of lentiviral particles into their local geography and institution.

In the U.S., download the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition, Feb 2007 here: cdc.gov/biosafety/publications/index.htm

See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), downloadable here: osp.od.nih.gov/biotechnology/nih-guidelines

For Biosafety Considerations for Research with Lentiviral Vectors, see osp.od.nih.gov/wp-content/uploads/Lenti_Containment_Guidance

Protocol I – plasmid preparation

Culture conditions for individual plasmid preparations

For plasmid preparation, grow all Precision LentiORF clones at 37 °C in 2x LB broth (low salt) medium plus 100 µg/mL carbenicillin.

2x LB broth (low salt) medium preparation:

LB-Broth-Lennox (Fisher Scientific Cat #BP1427500)	20 g/L
Peptone(Fisher Scientific Cat #BP9725–2)	10 g/L
Yeast Extract (Fisher Scientific Cat #BP1422–500)	5 g/L
Carbenicillin (Fisher Scientific Cat #BP2648–250)	100 µg/mL

Most plasmid miniprep kits recommend a culture volume of 1–10 mL for good yield.

1. Upon receiving your glycerol stock(s) containing the ORF of interest store at –80 °C until ready to begin.
2. To prepare plasmid DNA, first 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 a 10 µL inoculum from the glycerol stock into 3–5 mL of 2x LB broth (low salt) medium with 100 µg/mL carbenicillin. Return the glycerol stock(s) to –80 °C.
4. Note: If a larger culture volume is desired, incubate the 3-5 mL culture for 8 hours at 37 ˚C with shaking and use as a starter inoculum. Dilute the starter culture 1:500-1:1000 into the larger volume.
5. Incubate at 37 °C for 18-19 hours with vigorous shaking.
6. Pellet the 3–5 mL culture and begin preparation of plasmid DNA.

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

Culture conditions for 96-well bio-block plasmid preparation

1. Prepare a 96-well bio-block with 1 mL per well of 2x LB broth (low salt) medium containing 100 µg/mL carbenicillin.
2. Inoculate each well with 1 µL of the glycerol stock culture.
3. Incubate at 37 °C with shaking (~ 170–200 rpm) for 18–19 hours to produce good plasmid yield. For plasmid preparation, follow the protocol recommended by the plasmid isolation kit manufacturer.

96-well bio-block plasmid preparation protocol in conjunction with an appropriate 96-well plasmid purification method are used to purify plasmid DNA. Combine the cultures from two bio-blocks prior to purification, and elute with water at the final step.

Protocol II – Precision LentiORF plasmid DNA

Analysis by restriction digest

The following is a sample protocol for restriction analysis of **Precision LentiORF plasmid DNA** using Thermo Scientific™ FastDigest™ EcoRI Restriction Enzyme (Cat #FD0274). EcoRI cuts 15 bases upstream of attB1 site flanking the ORF at the 5' end (which corresponds to 30-50 nt upstream of ORF start codon, depending on the clone configuration – see specific clone information). A second EcoRI site is located 1348 bp downstream of attB2 site, approximately 1.37 kb downstream of the last sense codon of the ORF. Alternatively, SpeI or PacI *flanking attB1* site at the 5' end and NheI or AscI *flanking attB2* at 3' end in the vector may be used to cut out the fragment corresponding to the ORF. Use of EcoRI is preferable for smaller ORFs.

1. Set up EcoRI restriction digest reaction as follows (Table 3).
2. Mix gently by pipetting.
3. Incubate at 37 °C for 5 minutes to digest.
4. Run cut and uncut plasmid on 1% agarose gel (Figure 3).

Table 3. Restriction digest reaction setup.

Component	Amount
Water, nuclease-free (Cat #R0581)	X µL
10x Thermo Scientific™ FastDigest™ buffer	2 µL
DNA sample (up to 1 µg) in water	X µL
FastDigest EcoRI (Cat #FD0274)	1 µL
Final volume	20 µL

Protocol III – tagging Dharmacon precision LentiORF with a C-terminal tag

Precision LentiORFs are available in "open" (the native stop codon has been removed) and "closed" (the ORF retains its native stop codon) configurations (see clone information). Translation of the "closed" configuration ORFs terminates at its natural stop codon immediately after last sense codon of the ORF reference sequence.

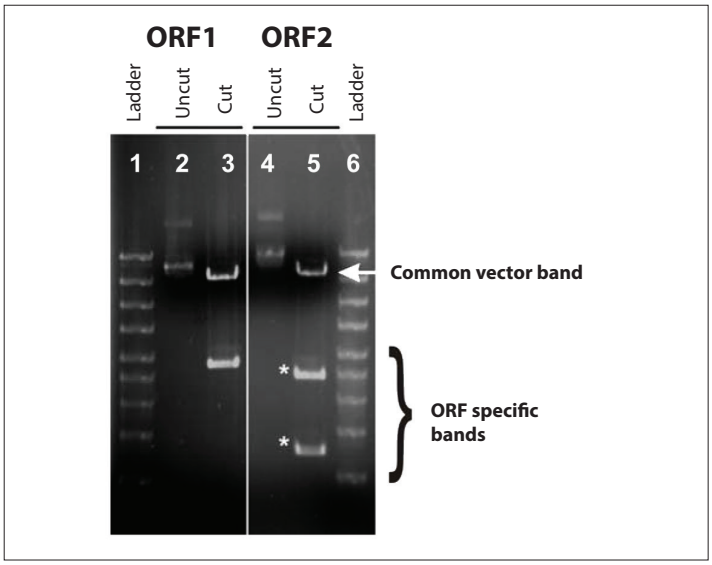

Figure 3. Restriction analysis of two Precision LentiORF clones with differen sized ORFs. Lanes 1 & 6 are a 10 kb molecular weight ladder (10 kb, 7 kb, 5 kb, 4 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb). Lanes 2 and 4 show uncut plasmid. Lanes 3 and 5 are EcoRI digests. The white arrow points to the 7.8 kb band common to the back bone of all Precision LentiORF clones. The lower bands (ORF1 producing a 2.8 kb band and ORF2 producing two bands, 2.5 kb and 1.3 kb) are specific to each ORF. ORF2 has an additional EcoRI site within it producing two ORF specific bands noted with the asterisks.

"Open" configuration of the ORFs was created to allow for C-terminal fusions of the ORFs with the tag of interest. In these ORFs, translation terminates at the stop codon in the vector immediately downstream of the attB2 site adding 11–16 amino acids to its C-terminus (Figure 4). If desired, the stop codons in the vector can be replaced by the fusion tag at Nhe/AscI sites flanking the stop codon in the vector. Care should be taken to place the fusion tag in the same reading frame as the stop codon.

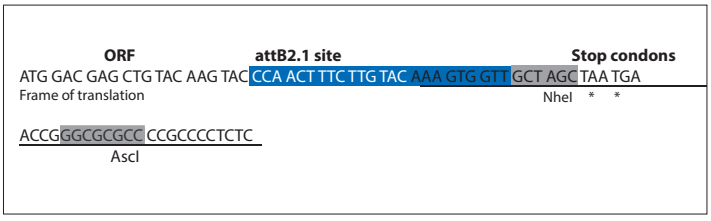

Figure 4. Example of 3' end of ORF and flanking sequence. Highlighted gray are NheI and AscI restriction sites flanking ORF-attB2 site at the 3' end. Highlighted blue is the attB2 site (version attB2.1) Underlined is an invariable part of the attB2 site and the adjacent vector sequence. The secondary stop codons have asterisks beneath them.

Protocol IV – Blasticidin S selection

Blasticidin S kill curve and selection

The pLOC vector confers resistance to Blasticidin S in transduced or transfected cells. Blasticidin S selection can be used to eliminate non-transduced or non-transfected cells. In order to generate stable cell lines, it is important to determine the minimum amount of Blasticidin S required to kill non-transfected or non-transduced cells.

We recommend testing your cell line of choice by generating a Blasticidin S kill curve following the protocol below. The general concentration range for many cells is 5–15 µg/mL.

Blasticidin S kill curve

1. On day 0, plate 5×10^4 cells per well in a 24-well plate in enough wells to carry out your Blasticidin S dilutions. Incubate overnight.
2. Prepare medium specifically for your cells containing a range of antibiotic, for example: 0–20 µg/mL Blasticidin S.
3. The next day (day 1) replace the growth medium with the medium containing dilutions of the antibiotic into the appropriate wells.
4. Incubate at 37 °C, 5% CO_2.
5. Approximately every 2-3 days replace with freshly prepared selective medium.
6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 3–6 days under Blasticidin S selection.
7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 3–6 days from the start of antibiotic selection.

Protocol V – transfection

For transfection of Precision LentiORF plasmids we recommend using DharmaFECT kb. Conditions should be optimized for your cell line of interest. For more details see our protocol.

Protocol VI – packaging lentivirus

The pLOC vector is tat dependent, so you must use a packaging system that expresses the *tat* gene. For packaging lentiviral constructs, we recommend the Trans-Lentiviral Packaging System. 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 of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the translentiviral system developed by Kappes *et al*[2]. For protocols and information on packaging Precision LentiORFs with our Trans-Lentiviral Packaging System, please see the product manual available on our website. Here

Protocol VII – viral titering

Viral titering

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice if you have produced viral particles yourself. This protocol uses the Dharmacon HEK293T cell line that is available as part of our Trans-Lentiviral ORF Packaging Kit (Cat #TLP5918). You can use a standard Dharmacon HEK293T cell line as an alternative.

If you have generated a lentiviral stock of the expression control (such as Precision LentiORF RFP control), we recommend titering this stock as well.

1. The day before transduction, seed a 24-well tissue culture plate with HEK293T cells at 5×10^4 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 serum-free medium. Utilize the plate as shown in Figure 5 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 five-fold dilutions to reach a final dilution of 390,625-fold.
3. To each well add 80 µL of serum-free medium.
4. Add 20 µL of thawed virus stock to each corresponding well in column 1 (five-fold dilution). Pipette contents of well up and down 10-15 times. Discard pipette tip.
5. With new pipette tips, transfer 20 µL from each well of column 1 to the corresponding well in column 2. Pipette contents of well up and down 10–15 times. Discard pipette tip.
6. Repeat transfers of 20 µL from columns 2 through 8, pipetting up and down 10–15 times and changing pipette tips between each dilution. Pre-incubate the dilutions of the virus stock for 5 minutes at room temperature.
7. Label the 24-well plate as shown in Figure 6 using one row for each virus stock to be tested.

It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions.

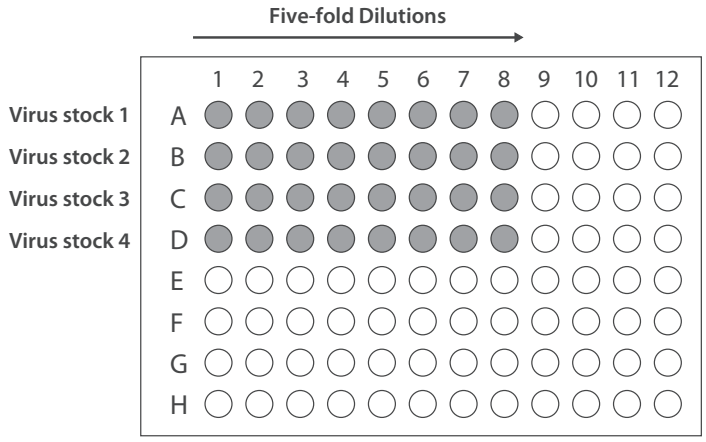

Figure 5. Five-fold serial dilutions of virus stock.

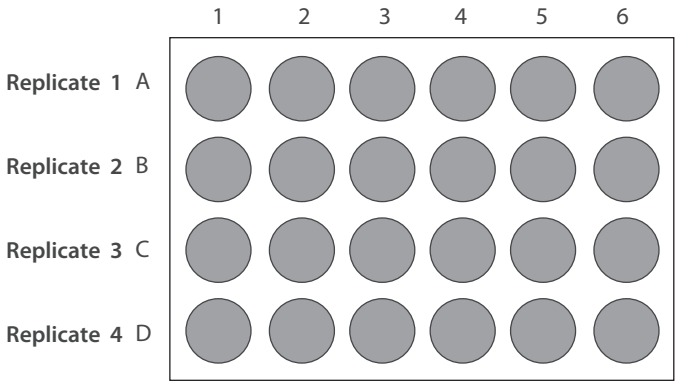

Figure 6. A twenty-four well tissue culture plate seeded with Dharmacon HEK293T cells is used to titer the virus.

8. Remove culture medium from the cells in the 24-well plate.
9. Add 225 µL of serum-free medium to each well.
10. Transduce cells by adding 25 µL of diluted virus from the original 96-well plate (Figure 5) to a well on the 24-well destination plate (Figure 6) 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 4).**
11. Incubate transduced cultures at 37 °C for 4 hours.
12. Remove transduction mix from cultures and add 1 mL of DMEM (10% FBS, 1% pen-strep).
13. Culture cells for 48 hours.
14. 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 7).

The intensity of TurboGFP may vary between LentiORF clones. If the TurboGFP intensity in your cells is low, we recommend counting colonies at a higher magnification.

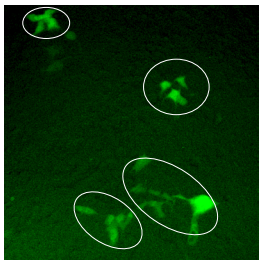

Figure 7. Example of individual colony 72 hour post-transduction. Four colonies are circled. Image taken at 40x magnification.

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

25 µL of diluted virus was added to the cells. This is 1/40th of a mL.

Example: 55 TurboGFP positive colonies counted in well A3. (TurboGFP positive colonies) × 625 (dilution factor) × 40 = 1.38 × 10^6 TU/mL

10^6 can be expected from non-concentrated lentiviral particles.

Table 4. 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 *

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, it generally correlates with the number of integration events per cell and, as a result, level of transgene expression.

Determining the optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cells (actively versus non-dividing), its relative 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 (for example, 0, 0.5, 1, 2, 5, 10, 20) to determine the MOI required to obtain optimal expression for your particular application. If minimal expression is preferred, it should be noted that to achieve single copy expression, an MOI of 0.3 is generally used followed by antibiotic selection, which typically results in less than 4% of your cells having more than one insert.

Protocol VIII - Transduction

Transduction of target cells

The protocol below is optimized for transduction of the lentiviral particles into Dharmacon HEK293T, OVCAR8 or MCF7 cells in a 24-well plate using serum-free medium. 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 5). It is strongly recommended that you optimize transduction conditions to suit your target cell line for the highest transduction efficiency possible.

It is preferable that transduction be carried out in medium that is serum-free and antibiotic-free. A reduction in transduction efficiency may occur in the presence of serum. However, it is possible to carry out successful transductions with serum present; you will need to optimize the protocol according to your needs.

1. On day 0, plate 5 × 10^4 cells per well in a 24-well plate. Incubate overnight.
2. The next day (day 1), remove the medium and add the virus to the MOI you wish to use. Set up all desired experiments and controls in a similar fashion. Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free medium (see Table 5 for guidelines).

If you are using concentrated virus you are likely to use very little virus volume and a lot of serum-free medium; if you are using unconcentrated virus you will find you need much more virus volume.

3. Approximately 4-6 hours post-transduction, add 1 mL of complete medium (serum plus pen-strep if you are using it) to your cells and incubate overnight.

We have experienced low toxicity with transduction in the cell lines tested, therefore removal of virus is not required for many cell lines. In our hands higher transduction efficiencies have been achieved if the virus is not removed after 6 hours. However, if toxicity is a problem, aspirate the mixture after 4-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

At 48 hours post-transduction examine the cells microscopically for the presence of reporter expression as this will be your first indication as to the efficiency of your transduction (Figure 8).

When visualizing Evrogen TurboGFP expression, if less than 90% of all cells are green, it is recommended to utilize Blasticidin S selection in order to increase the percentage of cells expressing your gene of interest.

a. If adding *Blasticidin S*, use the appropriate concentration as determined based on the kill curve experiment. If the cell density is close to or above 50% confluency, it is recommended that you split the cells at the time of Blasticidin S addition.
b. Approximately every 2–3 days replace with freshly prepared selective medium.
c. Monitor the cells daily and observe the percentage of surviving cells. At some time point almost all of the cells surviving selection will be harboring the gene of interest. Optimum effectiveness should be reached in 3–6 days with Blasticidin S, at which time all the cells surviving selection will harbor the gene of interest.

The higher the MOI that is used the more copies of the gene of interest and Blasticidin S resistance gene you will have per cell. When selecting on Blasticidin S, it is worth remembering that at higher MOIs, cells containing multiple copies of the resistance gene can withstand higher Blasticidin S concentrations than those at lower MOIs. Even at single copy the marker in the pLOC vector provides significant resistance to Blasticidin S in transduced cells. Cells with very low transgene expression level may still survive the selection even if TurboGFP is expressed at an undetectable level (Figure 8). We recommend using untransduced control cells to monitor the selection process. Longer selection and higher concentrations of Blasticidin S are recommended if high level of transgene expression is desired. Adjust the concentration of Blasticidin S 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.

Table 5. Suggested volumes of medium per surface area per well of adherent cells.

Tissue culture dish	Surface area per Well (cm^2)	Suggested total serum-free medium volume per well (mL)
100 mm	56.0	5.0
60 mm	20.0	2.0
35 mm	8.0	1.0
6-well	9.4	1.0
12-well	3.8	0.5
24-well	1.9	0.25
96-well	0.3	0.1

4. Once observed transduction efficiency is at an acceptable level (with or without Blasticidin S selection), you can proceed to assay cells for gene expression by reverse transcription quantitative real-time PCR (RT-qPCR), western blot analysis or other appropriate functional assay. The Evrogen TurboRFP expression construct may be used as a control.

Optimal length of incubation from the start of transfection/ transduction to analysis is dependent on cell type and the specific gene being over expressed.

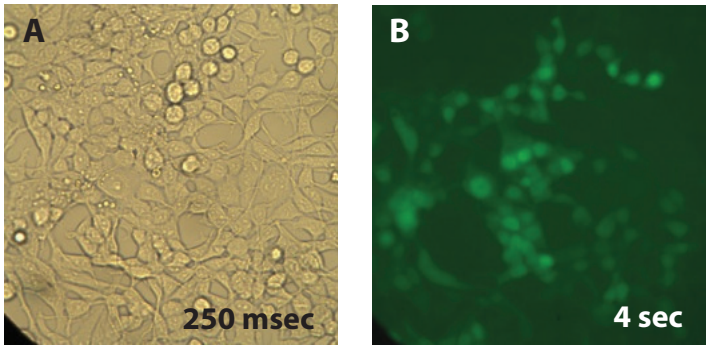

Figure 8. HEK293T cells transduced with Precision LentiORF expressing ANGPT1 HEK293T cells were plated at 200,000 cells/well (6-well format) and the next day transduced at MOI 0.3. Two days after transduction, the cells were selected with Blasticidin S (2.5 µg/mL) for 5 days. **A.** Phase contrast. **B.** TurboGFP expression.

Protocol IX – determining relative transduction efficiency

Follow the procedure below to determine the relative transduction efficiency of purchased Dharmacon Precision LentiORF viral particles. This protocol should be used with purchased Precision LentiORF RFP control in viral particle format.

Prior to transducing with purchased Precision LentiORF individual clones in viral particle format, we recommend determining the relative transduction efficiency of your cell type. Lentiviral titers provided with purchased Precision LentiORF viral particles have been calculated by transducing Dharmacon HEK293T cells. Transduction efficiencies vary significantly by cell type. The relative transduction efficiency of your cells may be estimated by determining the functional titer of a control virus such as the Precision LentiORF TurboRFP control viral particles (Cat #OHS5833) in your cells of interest.

Follow the procedure below to determine the functional titer of the LentiORF viral stock in the mammalian cell line of your choice. The following conditions have been optimized for transduction of Dharmacon HEK293T cells. When determining the relative transduction efficiency of your cell type, use the transduction conditions that have been optimized for your cell type of choice.

1. The day before transduction, seed a 24-well tissue culture plate with your cells at ~ 5 × 10^4 cells per well in their respective medium.

The following day, the well should be no more than 40-50% confluent.

2. Make dilutions of the Dharmacon LentiORF control viral stock in a round bottom 96-well plate using serum-free medium. Utilize the plate as shown in Figure 9 with one row for each replicate (we recommend performing at least two replicates). Use the procedure below for dilution of the viral stock. The goal is to produce a series of five-fold dilutions to reach a final dilution of 390,625-fold (Figure 5).

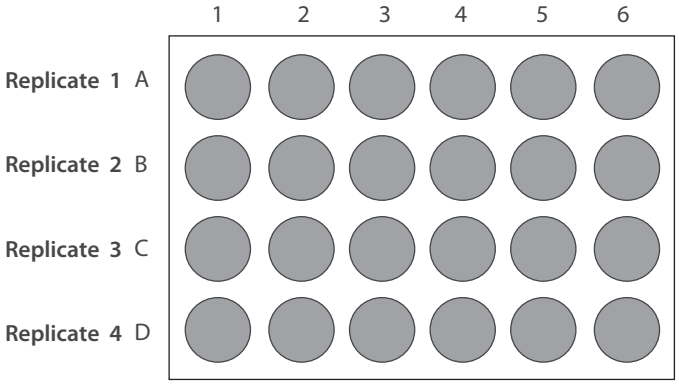

Figure 9. A twenty-four well tissue culture plate seeded with desired cells is used to titer the virus.

3. Add 40 µL of serum-free medium to each well in column 1. Add 80 µL of serum-free medium to each well of columns 2-8. If desired, include 8 µg/mL polybrene in the dilution medium.
4. Add 10 µL of thawed control virus stock to each well in column 1 (five-fold dilution). Pipette contents of well up and down 10–15 times. Discard pipette tip.
5. With new pipette tips, transfer 20 µL from each well of column 1 to the corresponding well in column 2. Pipette contents of well up and down 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 contents of well up and down 10–15 times and discard pipette tips.
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. Pre-incubate the dilutions of the virus stock for 5 minutes at room temperature.

It is strongly recommended that you use a high quality multichannel pipettor when performing multiple dilutions.

8. Label the 24-well plate as shown in Figure 9 using one row for each replicate.
9. Remove culture medium from the cells in the 24-well plate.
10. Add 225 µL of serum-free medium to each well.
11. Transduce cells by adding 25 µL of diluted control LentiORF lentivirus from the original 96-well plate (Figure 5) to a well on the 24-well destination plate (Figure 9) 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 4).
12. Incubate transduced cultures at 37 °C for 4–6 hours.
13. Add 1 mL of your medium (normal serum concentration).
14. Culture cells for 72 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 72 hour culture period (Figure 7). Count the number of TurboGFP expressing colonies in wells corresponding to at least two viral dilutions.

The intensity of TurboGFP may vary between LentiORF clones. If the TurboGFP intensity in your cells is low, we recommend counting colonies at a higher magnification.

Transducing units per mL (TU/mL) can be determined using the following formula:

# of TurboGFP positive colonies counted × dilution factor × 40 = # TU/mL

25 µL of diluted virus was added to the cells. This is 1/40th of a mL.

Example: 55 TurboGFP positive colonies counted in well A6.
55 (TurboGFP positive colonies) x 78,125 (dilution factor) x 40 = 1.72 x 10^8 TU/mL

16. The functional titer calculated for your cell line under your experimental conditions can be used to determine the relative transduction efficiency of your cell type by using the following formula:
    Functional titer of Dharmacon Precision LentiORF RFP control virus stock in your cell line
    ÷ Titer of LentiORF RFP control virus stock as calculated by Dharmacon in HEK293T
    = Relative transduction efficiency

For example, if the titer of the LentiORF RFP control virus stock in HEK293T (as provided on the product specification sheet) is 6.9 x 10^8 TU/mL and the functional titer of the LentiORF RFP control virus stock in your cell line is 1.72 x 10^8 TU/mL, the relative transduction efficiency of your cell type is 0.25. For accurate MOI calculations in your cells of interest, multiply the provided titer for each construct by the relative transduction efficiency determined for your cells of interest.

Once the relative transduction efficiency of the virus has been established in your cell line, use the optimized transduction conditions determined in Protocol VIII to transduce your cell line with the purchased Precision LentiORF individual clones in viral particle format. If the titer of the control virus is not satisfactory in your cell line you might consider choosing a different cell line more permissive to transduction by lentivirus before proceeding.

Controls and validation

Precision LentiORF starter kits

The use of vector-based ORF expression is a powerful and versatile tool. Successful gene expression in vitro is dependent on several variables including:

1) The target cell line being studied, 2) Transfection and transduction efficiency, 3) Abundance of the endogenous protein of interest in the target cell line, and 4) Robust experimental protocols. For all these reasons it is very important to run controlled experiments where the transfection and transduction efficiencies are as high as possible and measurable.

Controls

The Precision LentiORF TurboRFP control has been validated for ORF expression experiments (Figure 10). This control has been tested in transfection- and transduction-based experiments for TurboRFP expression and selection in Blasticidin S.

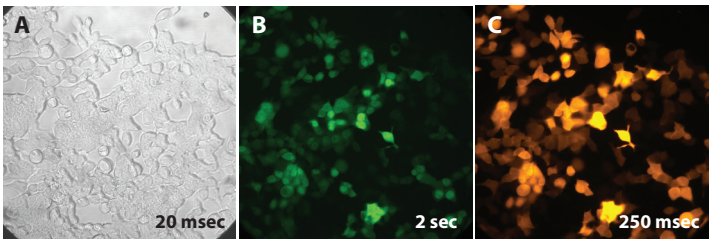

Figure 10. The HEK293T cells transduced with pLOC-TurboRFP lentiviral particles. DharmaconHEK293T cells were plated at 7,000 cells/well in 200 µL of medium (96-well format). The next day cells were transduced at an MOI of 20 in serum-free medium for 5 hours before full serum medium was added. Cells were imaged at 72 hours after transduction (40x magnification).

A. Phase contrast. **B.** Nuclear TurboGFP expression. **C.** TurboRFP expression.

Protocol X – replication

Replication from individual glycerol stock

Prepare 2X LB broth as directed in Protocol I with 8% glycerol* (Fisher Scientific Cat #BP2291) and the appropriate antibiotics, and inoculate from glycerol stock. For archive replication, grow all Precision LentiORF clones at 37 °C in LB-Lennox (low salt) medium plus 100 µg/mL carbenicillin in order to provide maximum stability of the clones.

Replication of plates

Prepare target plates by dispensing ~ 160 µL of 2x broth (low salt) medium supplemented with 8% glycerol* and appropriate antibiotic (100 µg/mL carbenicillin).

Prepare source plates

1. Remove foil seals (Fisher Scientific Cat #12-565–475) while the source plates are still frozen to minimize cross-contamination.
2. Thaw the source plates with the lid on. Wipe any condensation underneath the lid with a paper wipe soaked in ethanol.

Replicate

1. Gently place a disposable replicator (Fisher Scientific Cat #NC9584102) in the thawed source plate and lightly move the replicator around inside the well to mix the culture. Make sure to scrape the bottom of the wells.
2. Gently remove the replicator from the source plate and gently place in the target plate and mix in the same manner to transfer cells.
3. Dispose of the replicator.
4. Place the lids back on the source plates and target plates.
5. Repeat steps 1–4 until all plates have been replicated.
6. Seal and return the source plates to the -80 °C freezer.
7. Place the inoculated target plates in a 37 °C incubator for 18–19 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.

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

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

FAQS/troubleshooting

For answers to questions that are not addressed here, please email technical support at ts.dharmacon@horizondiscovery.com

What packaging system should I use for making lentivirus?

The pLOC vector is tat dependant, so you must use a packaging system that expresses the *tat* gene. For packaging our lentiviral constructs, we recommend the Trans-Lentiviral Packaging System. The Trans-Lentiviral ORF Packaging System allows creation of a replication-incompetent[2], 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 Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes *et al*[2]. For protocols and information on packaging pLOC with our Trans-Lentiviral Packaging System, please see the product manual available on our website.

Can I use any 2nd generation packaging system with the pLOC vector?

The pLOC vector is tat-dependant, so you must use a packaging system that expresses the *tat* gene.

What does the number 40 refer to in the formula for the calculation of titer?

The titer units are given in transducing units (TU) per mL, so the number 40 is used to convert the 25 µL used in the titration ("volume of diluted virus used, (Table 5) to one milliliter.

How can I make a stable cell line?

In order to generate stable cell lines, it is important to determine the minimum amount of Blasticidin S required to kill non-transfected/transduced cells. This can be done by generating a Blasticidin S kill curve. After you have determined the appropriate concentration of Blasticidin S to use, you can transfect or transduce your cells with the construct and culture with Blasticidin S in order to select for those cells that have a stable integrant.

Where do you purchase Blasticidin S?

Blasticidin S is available from Fisher Scientific (Cat #BP2647–25).

If transduction into your cell line is unsuccessful, you may need to consider the following list of factors influencing successful transduction:

1. Transduction efficiency is integrally related to the quality and the quantity of the virus you have produced. Factors to consider when transducing include MOI (related to accurate titer), the presence of serum in the medium, the use of polybrene in the medium, length of exposure to virus, and viral toxicity to your particular cells.
2. High quality transfer vector DNA and the appropriate and efficient viral packaging are required to make high quality virus able to transduce cells effectively.
3. All cell lines are not equally permissible to transduction by lentivirus. You may consider testing additional cell lines to find one more suitable for your experiments.

References

1. X. Wu, J.K. Wakefield, Development of a novel trans-lentiviral vector that affords predictable safety. *Mol. Ther.* **2**, 47–55 (2000).
2. J.C. Kappes, X. Wu, Safety considerations in vector development. *Somat. Cell Mol. Genet.* **26**, 147–158 (2001).

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For more information

To find the contact information in your country for your technology of interest, please visit us at **horizondiscovery.com/contact-us**

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