Thermo Scientific Open Biosystems
Precision LentiORF Collection

Catalog #: OHS5830, OHS5831, OHS5897, OHS5898, OHS5899, OHS5900

Important Safety Note
Please follow the safety guidelines for use and production of vector-based lentivirus as set by your institution’s biosafety committee. In general, the NIH Office of Biotechnology BSL2 or BSL2+ guidelines should be followed. Additional information on the safety features incorporated in the Precision LentiORF expression vector and the Trans-Lentiviral packaging system can be found on page 2.

Please note that Precision LentiORF vectors are not compatible with third generation packaging systems such as ViraPower from Invitrogen. We recommend the Thermo Scientific Open Biosystems Trans-Lentiviral Packaging System for use with our vectors.

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

Product Description
The Precision LentiORF collection is a library of expression ready lentiviral ORFs generated using gene content from the fully sequenced ORFeome Collaboration Collection. Its unique design expresses the ORF, the fluorescent marker and the selection marker from one promoter, providing a visual marker for ORF expression and two methods of transduction optimization. When used in viral 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.

- Versatile vector design with convenient cloning site for the addition of fusion tags
- Efficient expression at low MOI
- Transfection or transduction options for ORF expression
- Dual marker cassette provides two ways to optimize transduction
  - Nuclear localized TurboGFP for easy tracking
  - Blasticidin 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

Table 1. Features Of The pLOC Vector

<table>
<thead>
<tr>
<th>ORF Cassette Elements</th>
<th>Lentiviral Elements</th>
<th>Plasmid Backbone Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV Promoter</td>
<td>SIN-LTR</td>
<td>pUC ori</td>
</tr>
<tr>
<td>ORF</td>
<td>5'LTR</td>
<td>AMP</td>
</tr>
<tr>
<td>MultiTag Cloning site</td>
<td>WRE</td>
<td></td>
</tr>
<tr>
<td>TurboGFP(nuc)</td>
<td>cPPT (Central Polypurine Tract)</td>
<td></td>
</tr>
<tr>
<td>2a-peptide signal</td>
<td>RRE (Rev Response Element)</td>
<td></td>
</tr>
<tr>
<td>Blast'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA Polymerase II promoter</td>
<td>Increases safety - 3' Self inactivating long terminal repeat</td>
<td>High copy replication and maintenance in E.coli</td>
</tr>
<tr>
<td>ORF</td>
<td>5' long terminal repeat</td>
<td>Ampicillin bacterial selectable marker</td>
</tr>
<tr>
<td>Restriction site for purification or localization tag</td>
<td>Enhances the stability and translation of transcripts</td>
<td></td>
</tr>
<tr>
<td>Marker to track ORF expression</td>
<td>Increases translocation into the nucleus of non-dividing cells</td>
<td></td>
</tr>
<tr>
<td>Allows dual translation of BlastR and TurboGFP(nuc)</td>
<td>Increases efficient packaging full-length viral genomes</td>
<td></td>
</tr>
<tr>
<td>Mammalian selectable marker</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The specific sequence of the att site may vary between clones. Refer to the data file online for the specific sequence.
Figure 2. Detailed vector map of pLOC lentiviral vector.

**Antibiotic Resistance**

pLOC contains 2 resistance markers (Table 2).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (carbenicillin)</td>
<td>100 μg/ml</td>
<td>Bacterial selection marker (outside LTRs)</td>
</tr>
<tr>
<td>Blasticidin S</td>
<td>variable</td>
<td>Mammalian selectable marker</td>
</tr>
</tbody>
</table>

**Quality Control**

Each ORF identity has been sequence validated following the cloning process. The att site, MultiTag cloning site and secondary stop codons have also been sequence validated.

**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. The GIPZ shRNAmir lentiviral platform minimizes these hazards to the greatest degree by combining a disabled viral genome with the proprietary 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 the most essential genetic elements necessary for packaging and integration (e.g., 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 GIPZ shRNAmir lentiviral particles requires a packaging step during which the expression construct containing the silencing sequence is enclosed in a viral capsid. Gene functions that facilitate this process (e.g., encoded by the structural genes gag, pol, env, etc.) 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. 2000.

With these safety measures in place, GIPZ shRNAmir lentiviral particles can be employed in standard Biosafety Level 2 tissue culture facilities and should be treated with the same level of caution as any other potentially infectious agent. Any investigator who purchases Thermo Scientific 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.
Replication From Individual Glycerol Stock
Prepare media with 8% glycerol* and the appropriate antibiotics, and inoculate from glycerol stock. For archive replication, grow all pLOC clones at 37°C in LB-Lennox (low salt) media 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 LB-Lennox (low salt) media supplemented with 8% glycerol* and appropriate antibiotic (100 μg/ml carbenicillin).

Prepare Source Plates
1. Remove foil seals while the source plates are still frozen. This minimizes 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 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 plate of the well.
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. 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. 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 glycerol stock for each plasmid preparation.

*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 Preparation
Culture Conditions For Individual Plasmid Preparations
For plasmid preparation, grow all pLOC clones at 37°C in LB broth (low salt) media plus 100 μg/ml carbenicillin only.


See also: NIH Guidelines For Research Involving Recombinant DNA Molecules (NIH Guidelines), September 2009, downloadable here: http://oba.od.nih.gov/rdna/nih_guidelines_oba.html

For Biosafety Considerations for Research with Lentiviral Vectors, see http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html

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### Table 3. Materials for plate replication

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-Lennox Broth (low salt)</td>
<td>VWR</td>
<td>EM1.00547.0500</td>
</tr>
<tr>
<td>Peptone, granulated, 2 kg - Difco</td>
<td>VWR</td>
<td>90000-368</td>
</tr>
<tr>
<td>Yeast Extract, 500 g, granulated</td>
<td>VWR</td>
<td>EM1.03753.0500</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sigma</td>
<td>S-3014</td>
</tr>
<tr>
<td>Glycerol</td>
<td>VWR</td>
<td>EM-2200 or 80030-956</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>Novagen</td>
<td>69101-3</td>
</tr>
<tr>
<td>Blasticidin S</td>
<td>Invivogen</td>
<td>ant-zn-5p</td>
</tr>
<tr>
<td>96-well microplates</td>
<td>Nunc</td>
<td>260860</td>
</tr>
<tr>
<td>Aluminum seals</td>
<td>Nunc</td>
<td>276014</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Genetix</td>
<td>X5034</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Scinomix</td>
<td>SCI-5010-OS</td>
</tr>
</tbody>
</table>
2X LB broth (low salt) media preparation

- LB-Broth-Lennox 20 g/l
- Peptone 10 g/l
- Yeast Extract 5 g/l

Appropriate antibiotic(s) at recommended concentration(s)

Most plasmid mini-prep kits recommend a culture volume of 1–10 ml for good yield.

1. Upon receiving your glycerol stock(s) containing the shRNAmir 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 (low salt) with 100 μg/ml carbenicillin. Return the glycerol stock(s) to -80°C.
   *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.*
4. Incubate at 37°C for 18-19 hours with vigorous shaking.
5. Pellet the 3-5 ml culture and begin preparation of plasmid DNA.
6. Run 3-5 μl of the plasmid DNA on a 1% agarose gel. The size of the pLOC vector containing your ORF will vary depending on the length of the ORF. It can range from <500 nt to >4 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 for each plasmid preparation.*

Culture Conditions For 96-Well Bio-Block Plasmid Preparation

Inoculate a 96-well bio-block containing 1 ml per well of 2X-LB (low salt) media with 100 μg/ml carbenicillin with 1 μl of the glycerol stock culture. Incubate at 37°C with shaking (~170-200 rpm). We have observed that incubation times between 18-19 hours produce good plasmid yield. For plasmid preparation, follow the protocols recommended by the plasmid isolation kit manufacturer.

*Note: 96-well bio-block plasmid preparation protocol in conjunction with a Qiagen Turbo kit (Catalog # 27191) are used to purify plasmid DNA. 2 bio-blocks are combined, the optional wash step is not performed and water is used for the elution.*

Protocol III - Precision Lentiorf Plasmid DNA Analysis By Restriction Digest

The following is a sample protocol for restriction analysis of *Precision Lentiorf* plasmid DNA using *EcoRI* restriction enzyme. *EcoRI* cuts 15 bases upstream of *attB1* site flanking the ORF at 5' (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’ and *NheI* or *AscI* flanking *attB2* at 3’ 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 reaction as follows (Table 4).
2. Mix gently by pipetting.
3. Incubate at 37°C for 1 hour to digest
4. Run cut and uncut plasmid in 1% agarose gel (Figure 3).

### Table 4. PCR Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, nuclease-free water</td>
<td>X μl</td>
</tr>
<tr>
<td>NEBuffer <em>EcoRI</em> (10X)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>DNA sample (500 ng) in water</td>
<td>X μl</td>
</tr>
<tr>
<td><em>EcoRI</em>, 10 U/μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20.0 μl</td>
</tr>
</tbody>
</table>

Protocol IV – Tagging Lentiorf With C-Terminal Tag

pLOC-ORF 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.
“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 (see figure 4 below). If desired the stop codons in the vector can be replaced by the fusion tag at NheI/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.

**Protocol V - Blasticidin S Selection**

**Blasticidin S Kill Curve and Blasticidin S Selection**
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 have tested several cell lines for their sensitivity to Blasticidin S (see Table 5).

These data can be used as a general guidelines. However, we recommend testing your cell line of by generating a Blasticidin S kill curve following the protocol below.

**Blasticidin S Kill Curve**
1. On day 0, plate $5 \times 10^4$ cells per well in a 24-well plate in enough wells to carry out your Blasticidin S dilutions. Incubate overnight.
2. Prepare media 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 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 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 VI - Transfection**
The protocol below is optimized for transfection of the pLOC-ORF 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 6).

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 Express-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 Express-In mixtures as they will be added to the cells.

1. The day before transfection (day 0), plate the cells at a density of 5 x 10⁴ cells per well of a 24-well plate.

2. On the day of transfection, form the DNA/Express-In transfection complexes.
   a. For each well to be transfected, dilute 500 ng ORF 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.

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 5-6 hours in a CO₂ incubator at 37°C. Your total volume will be 250 µl at this stage (150 μl serum-free medium + 100 μl DNA:Express-In mixture).

4. Following the 5-6 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 CO₂ 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 5-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

5. After 24-72 hours of incubation, examine the cells microscopically for the TurboGFP expression.

   Note: If desired, Blasticidin S selection can be applied 24 h post transfection. The working concentration of Blasticidin S varies between cell lines. We recommend you determine the optimal concentration of Blasticidin S required to kill your host cell line prior to selection for pLOC-ORF transfectants (see Blasticidin S kill curve protocol). Typically, the working concentration ranges from 5-20 µg/ml. We recommend that you change selection media every 2-3 days. You should use the lowest concentration that kills 100% of the cells in 3-6 days from the start of Blasticidin S selection.

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**Table 6.** Suggested amounts of DNA, medium and Express-In reagent for transfection of shRNA plasmid DNA into adherent cells.

<table>
<thead>
<tr>
<th>Tissue Culture Dish</th>
<th>Surface Area per Plate or Well (cm²)</th>
<th>Total Serum-Free Media Volume per Well (ml)</th>
<th>Plasmid DNA (µg)*</th>
<th>Arrest-In (µg)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mm</td>
<td>20.0</td>
<td>2.0</td>
<td>4.0</td>
<td>21.0</td>
</tr>
<tr>
<td>35 mm</td>
<td>8.0</td>
<td>1.0</td>
<td>2.0</td>
<td>10.0</td>
</tr>
<tr>
<td>6-well</td>
<td>9.4</td>
<td>1.0</td>
<td>2.0</td>
<td>10.0</td>
</tr>
<tr>
<td>12-well</td>
<td>3.8</td>
<td>0.5</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>24-well</td>
<td>1.9</td>
<td>0.25</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>96-well</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1 - 0.2</td>
<td>0.5 - 1.0</td>
</tr>
</tbody>
</table>

*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.**
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 Thermo Scientific Open Biosystems Express-In and DNA amount indicated in Table 6 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.

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/Open Biosystems 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).

Protocol VII - Packaging Lentivirus

The pLEX vector is tat dependant, 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 (Shimada, et al. 1995), 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 (Kappes and Wu 2001). For protocols and information on packaging pLEX with our Trans-Lentiviral pLEX Packaging System, please see the product manual available on our website.

Protocol VIII - 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 HEK293T cell line that is available as part of our Trans-Lentiviral shRNA 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. pLOC Non-Silencing), we recommend titering this stock as well.

1. The day before transduction, seed a 24-well tissue culture plate with 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. HEK293T (Thermo Scientific Open Biosystems Catalog # HCL4517).

2. Make dilutions of the viral stock in a round bottom 96-well plate using serum-free media. Utilize the plate as shown in Figure 6 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.
3. To each well add 80 μl of serum-free media.

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.

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 7 using one row for each virus stock to be tested.

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

10. Add 225 μl of serum-free media to each well.

11. Transduce cells by adding 25 μl of diluted virus from the original 96-well plate (Figure 6) to a well on the 24-well destination plate (Figure 7) 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 7).

12. Incubate transduced cultures at 37°C for 4 hours.
13. Remove transduction mix from cultures and add 1 ml of DMEM (10% FBS, 1% pen-strep).

14. Culture cells for 48 hours.

15. Count the TurboGFP expressing cells or colonies of cells (Figure 8).

Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 8 illustrates this principle of counting. Note: 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.

16. Transducing units per ml (TU/ml) can be determined using the following formula:

\[
\text{# of TurboGFP positive colonies counted} \times \text{dilution factor} \times 40 = \text{#TU/ml}
\]

Example: 55 TurboGFP positive colonies counted in well A3.

\[
(\text{TurboGFP positive colonies}) \times 625 \times 40 = 1.38 \times 10^6 \text{ TU/ml}
\]

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 per cell 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- 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. If minimal expression is preferred, 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.

**Protocol IX - Transduction**

**Transduction Of Target Cells**

The protocol below is optimized for transduction of the lentiviral particles into HEK293T, OVCAR8 or MCF7 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 (see Table 8). It is strongly recommended that you optimize transduction conditions to suit your target cell line to provide for the highest transduction efficiency possible.

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**Table 7. Example of setup for dilutions**

<table>
<thead>
<tr>
<th>Well (Row A, B, C, or D)</th>
<th>Volume Diluted Virus Used</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Originating (96-well plate)</td>
<td>Destination (24-well plate)</td>
<td>25 μl</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>25 μl</td>
</tr>
<tr>
<td>A2</td>
<td>A1</td>
<td>25 μl</td>
</tr>
<tr>
<td>A3</td>
<td>A2</td>
<td>25 μl</td>
</tr>
<tr>
<td>A4</td>
<td>A3</td>
<td>25 μl</td>
</tr>
<tr>
<td>A5</td>
<td>A4</td>
<td>25 μl</td>
</tr>
<tr>
<td>A6</td>
<td>A5</td>
<td>25 μl</td>
</tr>
<tr>
<td>A7</td>
<td>A6</td>
<td>25 μl</td>
</tr>
<tr>
<td>A8</td>
<td></td>
<td>25 μl</td>
</tr>
</tbody>
</table>

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

---

Figure 8. Example of individual colony 72 h post transduction. Imaged at 40x magnification.
It is preferable that transduction be carried out in medium that is serum-free and antibiotic-free. A reduction in transduction efficiency occurs in the presence of serum, however it is possible to carry out successful transductions with serum present; you will have to optimize the protocol according to your needs.

1. On day 0 plate $5 \times 10^4$ cells per well in a 24-well plate. Incubate overnight.
   
   You will be using full medium (i.e. with serum) at this stage.

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 media (See Table 8 for guidelines). 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.

3. 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.
   
   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 3-6 hours and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

4. 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.
   
   Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases 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 above kill curve. 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. Incubate.
   
   b. Approximately every 2-3 days replace with freshly prepared selective media.
   
   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.

   Please note that the higher the MOI you have chosen 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 pLOC lentiORFs provides significant resistance to Blasticidin in transduced cells. Cells with very low transgene expression level may still survive the selection even if TurboGFP is expressed at an undetectable level (see Figure 9). 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.

5. Once your transduction efficiency is at an acceptable level (with or without Blasticidin S selection), you can proceed to assay cells for gene expression by quantitative/real-time PCR (QPCR), western blot or other appropriate functional assay. The 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 gene of interest.

---

Table 8. Suggested volumes of media per surface area per well of adherent cells.

<table>
<thead>
<tr>
<th>Tissue Culture Dish</th>
<th>Surface Area per Well (cm²)</th>
<th>Suggested total serum-free medium volume per well (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mm</td>
<td>56.0</td>
<td>5.0</td>
</tr>
<tr>
<td>60 mm</td>
<td>20.0</td>
<td>2.0</td>
</tr>
<tr>
<td>35 mm</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>6-well</td>
<td>9.4</td>
<td>1.0</td>
</tr>
<tr>
<td>12-well</td>
<td>3.8</td>
<td>0.5</td>
</tr>
<tr>
<td>24-well</td>
<td>1.9</td>
<td>0.25</td>
</tr>
<tr>
<td>96-well</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 9. HEK293T cells after 4 days of selection on Blasticidin S at concentration 10μg/ml. a) untransduced control. The rounded cell morphology compared to the transduced cells is an indication of cell death for HEK293T cells, b) and c) cells transduced with pLOC-ORF viral particles (MOI=0.3) and placed on selection 24 hours post transduction. Imaged 5 days post transduction.

Protocol X – Determining Relative Transduction Efficiency

Follow the procedure below to determine the relative transduction efficiency of purchased LentiORF viral particles (Thermo Scientific Open Biosystems Catalog # OHS5831). This protocol should be used with purchased LentiORF RFP in viral particle format.

Prior to transducing with purchased LentiORF individual clones in viral particle format, we recommend determining the relative transduction efficiency of your cell type. Lentiviral titers provided with purchased LentiORF viral particles have been calculated by transducing TLAHEK293T 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 LentiORF TurboRFP control viral particles (Thermo Scientific Open Biosystems Catalog # OHS5831) in your cell type of choice.

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 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 5x10⁴ cells per well in their respective media.

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

2. Make dilutions of the LentiORF control viral stock in a round bottom 96-well plate using serum-free medium. Utilize the plate as shown in Figure 10 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 5-fold dilutions to reach a final dilution of 390625-fold.

![Figure 10. Five-fold serial dilutions of virus stock.](image-url)
3. Add 40 ul of serum-free media to each well in column 1. Add 80 ul of serum-free media to each well of columns 2-8.

   *If desired, include 8 μg/mL polybrene in the dilution media*

4. Add 10 µl of thawed control virus stock to each well in column 1 (5-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 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 a 24-well plate as shown in Figure 11 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 10) to a well on the 24-well destination plate (Figure 11) 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 9).*

12. Incubate transduced cultures at 37°C for 4-6 hours.

**Table 9. Example of set up for dilutions**

<table>
<thead>
<tr>
<th>Well (Row A, B, C, or D)</th>
<th>Volume Diluted Virus Used</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Originating (96-well plate)</td>
<td>Destination (24-well plate)</td>
<td>25 µl</td>
</tr>
<tr>
<td>A1</td>
<td>A1</td>
<td>25 µl</td>
</tr>
<tr>
<td>A2</td>
<td>A2</td>
<td>25 µl</td>
</tr>
<tr>
<td>A3</td>
<td>A3</td>
<td>25 µl</td>
</tr>
<tr>
<td>A4</td>
<td>A4</td>
<td>25 µl</td>
</tr>
<tr>
<td>A5</td>
<td>A5</td>
<td>25 µl</td>
</tr>
<tr>
<td>A6</td>
<td>A6</td>
<td>25 µl</td>
</tr>
<tr>
<td>A7</td>
<td>A7</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

*Please note that when expecting very high or very low transduction efficiency, it would be advisable to include either higher or lower dilution factors.*
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 6 illustrates this principle of counting. Count the number of TurboGFP expressing colonies in wells corresponding to at least two viral dilutions. Note: 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.

16. Transducing units per ml (TU/ml) can be determined using the following formula:

   \[ \text{# of TurboGFP positive colonies counted} \times \text{dilution factor} \times 40 = \text{# TU/mL} \]

   (Note: 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.
   \[ 55 \times 625 \times 40 = 1.38 \times 10^6 \text{ TU/ml} \]

17. 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:

   \[ \frac{\text{Functional titer of Non-silencing control virus stock in your cell line}}{\text{Titer of Non-silencing control shRNAmir virus stock as calculated by Thermo Scientific Open Biosystems in HEK293T}} = \text{Relative transduction efficiency} \]

   For example, if the titer of the non-silencing control shRNAmir virus stock in HEK293T (as provided on the product specification sheet) is \(6.9 \times 10^6\) TU/mL and the functional titer of the control shRNAmir virus stock in your cell line is \(1.38 \times 10^6\) TU/mL, the relative transduction efficiency of your cell type is 0.2. To extrapolate the average functional titer of the provided PLOC viral particles, multiply the average titer of each plate as provided on the product specification sheet by the relative transduction efficiency of your cell type. In our example, if the titer of the PLOC viral particles in HEK293T cells is \(2 \times 10^6\) TU/mL and the relative transduction efficiency of your cell type is 0.2, the extrapolated average functional titer of that plate your cell type is \(4 \times 10^5\) TU/mL.

   Once the relative transduction efficiency of the PLOC virus has been established in your cell line, use the optimized transduction conditions determined in Protocol IX to transduce your cell line with the purchased PLOC shRNAmir individual clones in viral particle format. If the titer of the non-silencing control shRNAmir 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 silencing *in vitro* is dependent on several variables including 1) The target cell line being studied 2) Transfection and transduction efficiency 3) Abundance of the mRNA or protein of interest in the target cell line 4) Half life of the protein 5) 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 are a critical part of a gene silencing experiment. They enable accurate representation of knockdown data and provide confidence in the specificity of the response. Changes in the mRNA or protein levels in cells treated with negative or non-silencing controls reflect nonspecific responses in cells and can be used as a baseline against which specific knockdown can be measured. Positive controls are useful to demonstrate that your experimental system is functional and your shRNA construct is successfully activating the RNAi pathway.

**Controls**

The pLOCTM TurboRFP lentiviral expression construct has been validated as control for ORF expression experiments. This control has been tested in transfection and transduction based experiments for TurboRFP expression and selection on Blasticidin S. Additional cell lines to the one below have been tested and are listed in Table 5.
What Packaging Cell Line 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 shRNAmir constructs, we recommend the Trans-Lentiviral Packaging System. The Trans-Lentiviral shRNA Packaging System allows creation of a replication-incompetent (Shimada, *et al*. 1995), HIV-1-based lentivirus which can be used to deliver and express your gene or shRNAmir of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes (Kappes and Wu 2001). For protocols and information on packaging pLOC with our Trans-Lentiviral shRNA 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 2.5 μl used in the titration (“volume of diluted virus used”, Table 8) 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. Cells not containing a stable integrant will not be selected for.

Where do you purchase Blasticidin S?
We purchase Blasticidin S from Cellgro™ (Catalog # 61-385-RA).

How Many Transfections Are Available In Each Volume Size Of Express-In?
The number of transfections that can be performed depends on the size of the culture dish used and the volume size of Express-In purchased. Refer to Table 10 below for the approximate number of transfections.

Table 10. Number of transfections depending on culture dish size and volume of Express-In purchased.

<table>
<thead>
<tr>
<th>Tissue Culture Dish</th>
<th>Surface Area per Well (cm²)</th>
<th>Arrest-In (1 mg/ml) (µg)</th>
<th>0.5 ml qty (rxns)</th>
<th>1.0 ml qty (rxns)</th>
<th>5.0 ml qty (rxns)</th>
<th>10 ml qty (rxns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mm</td>
<td>20.0</td>
<td>21.0</td>
<td>47-50</td>
<td>100</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>35 mm</td>
<td>8.0</td>
<td>10.0</td>
<td>100</td>
<td>200</td>
<td>1000</td>
<td>2000</td>
</tr>
<tr>
<td>6-well</td>
<td>9.4</td>
<td>10.0</td>
<td>100</td>
<td>200</td>
<td>1000</td>
<td>2000</td>
</tr>
<tr>
<td>12-well</td>
<td>3.8</td>
<td>5.0</td>
<td>200</td>
<td>400</td>
<td>2000</td>
<td>4000</td>
</tr>
<tr>
<td>24-well</td>
<td>1.9</td>
<td>2.5</td>
<td>400</td>
<td>800</td>
<td>4000</td>
<td>8000</td>
</tr>
<tr>
<td>96-well</td>
<td>0.3</td>
<td>0.5-1.0</td>
<td>1000</td>
<td>2000</td>
<td>10000</td>
<td>20000</td>
</tr>
</tbody>
</table>

**Recommended starting amounts of Arrest-In reagent as defined in Table 1.
**Approximate number of transfections based on recommended starting amount of Express-In. Individual results may vary depending on amounts of Express-In used.

1. How much time elapsed from transfection/transduction to Blasticidin S selection?
If Transfection Into Your Cell Line Is Unsuccessful, You May Need To Consider The Following List Of Factors Influencing Successful Transfection

1. Concentration and purity of plasmid DNA and nucleic acids—determine the concentration and purity of your DNA using 260/280 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.

2. Insufficient mixing of transfection reagent or transfection complexes.

3. Transfection in serum containing or serum free media—our studies indicate that Express-In/DNA complexes should preferably be formed in the absence of serum. In the cell lines tested we found that the highest transfection efficiencies can be obtained if the cells are exposed to the transfection complexes in serum free conditions followed by the addition of medium containing twice the amount of normal serum to the complex medium 3-6 hours post transfection (leaving the complexes on the cells). However, the serum-free transfection medium can be replaced with normal growth medium if high toxicity is observed.

4. Presence of antibiotics in transfection medium—the presence of antibiotics can adversely affect the transfection efficiency and lead to increased toxicity levels in some cell types. It is recommended that antibiotics be excluded until transfection has mostly occurred (3-6 hours) and then be added together with the full medium.

5. High protein expression levels—some proteins when expressed at high levels can be cytotoxic; this effect can also be cell line specific.

6. 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). Plate cells at a consistent density to minimize experimental variation. 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.

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 bear in mind when transducing include MOI (related to accurate titer), the presence of serum in the media, the use of polybrene in the media, 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. See also suggestions 3-6 for factors influencing successful transfection (above).

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.

If Express-In seems to be toxic to a particular cell line, try reducing the DNA:Express-In ratio.

FAQs/Troubleshooting

For answers to questions that are not addressed here, please email technical support at openbiosystems@thermofisher.com with your question, your sales order or purchase order number and the catalog number or clone ID of the construct or collection with which you are having trouble.
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