Expression Arrest™-The RNAi Consortium (TRC) lentiviral shRNA
RHS3979, RHS4012, RMM3981, RMM4013

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PRODUCT DESCRIPTION

The Expression Arrest TRC library is the result of a collaborative research effort based at the Broad Institute of MIT and Harvard, and includes six MIT and Harvard associated research institutions and five international life sciences organizations. The goal of TRC is to create lentiviral shRNA libraries targeting 15,000 human and 15,000 mouse annotated genes with multiple constructs per gene. Open Biosystems has partnered with the TRC to make these shRNA libraries available to researchers worldwide.

SHIPPING AND STORAGE

Individual constructs are shipped as bacterial cultures of E. coli (DH5α) in LB-Lennox (low salt) broth with 8% glycerol, 100µg/ml carbenicillin. Individual constructs are shipped on wet ice. Collections are shipped in 96 well plate format on dry ice. Individual constructs and collections should be stored at -80°C.

Open Biosystems checks all cultures for growth prior to shipment.

TO ALLOW ANY CO2 THAT MAY HAVE DISSOLVED INTO THE MEDIA FROM THE DRY ICE IN SHIPPING TO DISSIPATE, PLEASE STORE CONSTRUCTS AT –80°C FOR AT LEAST 48 HOURS BEFORE THAWING.

Important Safety Note:
Follow NIH guidelines regarding lentiviral production and transduction; follow Biosafety Level 2 (BL2) or BL2+ laboratory criteria.
DESIGN INFORMATION
The TRC library design:
The shRNA constructs were designed to include a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop. Each hairpin sequence was cloned into the lentiviral vector (pLKO.1) and sequence verified. Multiple constructs (4-5) were created per gene to ensure adequate coverage of the target gene. The TRC predicts that 1 or 2 out of the 4-5 constructs offered per gene are expected to give at least 70% knockdown.

Features of the TRC shRNA library include:
- Rules-based shRNA design for efficient gene knockdown
- Already cloned into lentiviral vectors
- Amenable to in vitro and in vivo applications such as the creation of stable cell lines
- Lentiviral vector enables transduction of primary and non-dividing cell lines
- Broad coverage: 4-5 constructs per gene

The TRC hairpin design:
Stem: 21 bases
Loop: 6 bases, XhoI restriction site: CTCGAG
Flanking = 5’ CCGG overhang for AgeI
3’ TTTTT termination for Pol III and AATT overhang for EcoRI

VECTOR INFORMATION
The pLKO.1 HIV-based lentiviral vector (Figures 1-2, Table 1) allows for transient and stable transfection of shRNA and also the production of viral particles using lentiviral packaging cell lines. Stable cell lines can be selected using the puromycin selectable marker.

Figure 1. The pLKO.1 vector

Table 1. Features of the pLKO.1 vector

<table>
<thead>
<tr>
<th>Vector Element</th>
<th>Utility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human U6 Promoter</td>
<td>RNA generated with four uridine overhangs at each 3’ end</td>
</tr>
<tr>
<td>hPGK</td>
<td>Human phosphoglycerate kinase promoter</td>
</tr>
<tr>
<td>PuroR</td>
<td>Puromycin mammalian selectable marker</td>
</tr>
<tr>
<td>3’ SIN LTR</td>
<td>3’ Self inactivating long terminal repeat</td>
</tr>
<tr>
<td>f1 ori</td>
<td>f1 origin of replication</td>
</tr>
<tr>
<td>AmpR</td>
<td>Ampicillin bacterial selectable marker</td>
</tr>
<tr>
<td>5’LTR</td>
<td>5’ long terminal repeat</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev response element</td>
</tr>
<tr>
<td>cPPT</td>
<td>Central polypurine tract</td>
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</table>
ANTIBIOTIC RESISTANCE

pLKO.1 contains 2 antibiotic resistance markers (Table 2). The TRC recommends the use of carbenicillin instead of ampicillin for the growth and maintenance of pLKO.1.

Table 2. Antibiotic Resistances Conveyed by pLKO.1

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

VECTOR MAP

Figure 2. Map of the pLKO.1 vector
PROTOCOLS
There are protocols recommended by the TRC for culturing, plasmid prep, virus production and transduction of TRC lentiviral shRNA constructs. These protocols can be accessed from the following link:

Culturing protocols and maintenance of pLKO.1
The Expression Arrest TRC shRNA Library is constructed in the pLKO.1 vector. This vector allows for both transient and stable gene knockdown via the mechanism of RNA interference. The vector is capable of producing self-inactivating lentiviral particles when used in conjunction with lentiviral packaging lines.

In order to obtain a good yield of cells in a short period of incubation, rich media containing carbenicillin and 8% glycerol should be used to culture pLKO.1 constructs. The TRC recommends the use of carbenicillin instead of ampicillin. An incubation period of 14-20 hours at 37°C with aeration is sufficient. It is recommended that the cultures remain frozen at –80°C when not in use. Freeze/thaw cycles do not seem to have any detrimental effect providing the cultures are not incubated at room temperature or higher, for long periods of time.

PROTOCOL I-REPLICATION

Table 3. Materials for plate replication

<table>
<thead>
<tr>
<th>Item</th>
<th>Vendor</th>
<th>Catalog Number</th>
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</thead>
<tbody>
<tr>
<td>LB-Lennox Broth (low salt)</td>
<td>VWR</td>
<td>EM1.00547.0500</td>
</tr>
<tr>
<td>Peptone, granulated, 2kg - Difco</td>
<td>VWR</td>
<td>90000-368</td>
</tr>
<tr>
<td>Yeast Extract, 500g, 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>VWR</td>
<td>EM-3130</td>
</tr>
<tr>
<td>Puromycin</td>
<td>Cellgro</td>
<td>61-385-RA</td>
</tr>
<tr>
<td>96 well microplates</td>
<td>VWR</td>
<td>62407-174</td>
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<tr>
<td>Aluminum seals</td>
<td>VWR</td>
<td>73520-056</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Genetix</td>
<td>X5054</td>
</tr>
<tr>
<td>Disposable replicators</td>
<td>Scinomix</td>
<td>SCI-5010-OS</td>
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2X-LB broth* (low-salt) media preparation

Peptone     20g/L  
Yeast Extract 10g/L  
NaCl        5g/L   
Carbenicillin 100µg/ml  
**Glycerol 8% for long term storage

* LB media can be used instead of 2XLB  
**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.

Replication of plates
Prepare target plates by dispensing ~160µl of LB media supplemented with 8% glycerol and appropriate antibiotic (100µg/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 14-20 hours.

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.

PROTOCOL II-PLASMID PREP
Culture conditions for individual plasmid preparations
Most plasmid mini-prep kits recommend a culture volume of 1–10ml for good yield. For shRNA constructs, 5ml of culture can be used for one mini-prep generally producing from 5–20µg of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNA 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. Using a sterile loop or a pipette tip, streak the shRNA culture onto a LB agar plate containing 100µg/ml carbenicillin. Incubate the plate overnight at 37°C. Return the glycerol stock(s) to –80°C.
4. The following day, pick 1 to 3 colonies from the agar plate and inoculate 6ml of the 2XLB. Incubate at 37°C for 16-20hrs with vigorous shaking (300rpm).
5. The following day remove 1ml of the culture and place in a sterile 2ml sterile microcentrifuge tube. Place this tube at 4°C until the plasmid DNA from the remaining culture has been analyzed. Pellet the remaining 5ml culture and begin preparation of plasmid DNA. We recommend preparing Ultra-pure DNA to ensure both high-purity and low endotoxin levels (Qiagen Catalog no.12123) as required for transfection into eukaryotic cells.

If you wish to continue at a later time cell pellets can be kept frozen at –20°C overnight.
6. Run 3-5µl of the plasmid DNA on a 1% agarose gel. The uncut pLKO.1 shRNA constructs run at about 7-10kb. Prepare an 8% glycerol stock culture using the 1ml of culture you removed prior to plasmid preparation. This culture can be used for future plasmid preparations but it is still recommended you streak isolate and work from a fresh colony. Store at –80°C.
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.

Gel images of plasmid isolated from cultures grown under the above conditions are shown below (Figure 3).

![Gel Image](image.png)

**Figure 3.** 1.5ml cultures of 92 different shRNA constructs after 20 hours of incubation at 37°C with shaking (≈170rpm). 2X LB media (low-salt) with 8% glycerol was used for culturing.

**PROTOCOL III- RESTRICTION DIGEST**

You may wish to restriction digest a sample of your plasmid DNA following plasmid DNA preparation. The following is a protocol for dual restriction enzyme digestion using BamHI and NdeI for quality control of pLKO.1 vectors.

Using filtered pipette tips and sterile conditions add the following components, in the order stated, to a sterile PCR thin-wall tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>Sterile, nuclease-free water</td>
<td>14.8µl</td>
</tr>
<tr>
<td>Restriction enzyme BamHI</td>
<td>1µl</td>
</tr>
<tr>
<td>Restriction enzyme BamHI 10X buffer</td>
<td>2µl</td>
</tr>
<tr>
<td>BSA (10X, 10mg/ml)</td>
<td>0.2µl</td>
</tr>
<tr>
<td>DNA sample 1µg, in water or TE buffer</td>
<td>1µl</td>
</tr>
<tr>
<td>Restriction enzyme NdeI 20U</td>
<td>1µl</td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td><strong>20µl</strong></td>
</tr>
</tbody>
</table>

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2.5 hours to digest then at 70°C for 20 minutes to kill the enzyme.
4. Add 4µl of 6X Loading Dye (or another appropriate DNA loading buffer), and proceed to gel analysis.
5. Load the gel with 20µl of the digested samples on a 1% agarose gel. Also run 1µl (1µg) of the uncut sample combined with 16µl of water and 3µl of 6x dye alongside the digested samples.
6. The digest will produce two fragments one approximately 6.3kb band and a 794bp band.
Figure 4. The 1% agarose gel above contains -10kb ladder followed by undigested sample and restriction digests of three TRC shRNA clones (lanes 2-9). The lanes are loaded as follows: 1 - Clone E1 Uncut plasmid 2 - Clone E1 Cut with BamHI. Expected to linearize at 7032bp. 3 - Clone E1 Cut with BamHI and NdeI. Band sizes of 6238bp and 794bp expected. 4-6 Repeat of 1-3 only with clone E2. 7-9 Repeat of 1-3 only with clone F1.

PROTOCOL IV-TRANSFECTION
The protocol below is optimized for transfection of the shRNA 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 4).

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

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

Table 4. Suggested amounts of DNA, medium and Arrest-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</td>
<td>2</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>35 mm</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>6 well</td>
<td>9.4</td>
<td>1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>12 well</td>
<td>3.8</td>
<td>0.5</td>
<td>1</td>
<td>5</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</td>
</tr>
</tbody>
</table>

*Recommended starting amount of DNA. May need to be optimized for the highest efficiency
**Recommended starting amount of Arrest-In reagent. See Transfection Optimization.
1. The day before transfection (day 0), plate the cells at a density of 5 x 10^4 cells per well of a 24 well plate.

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

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

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

a. For each well to be transfected, dilute 500ng shRNA plasmid DNA into 50µl (total volume) of serum free medium in a microfuge tube.

b. For each well to be transfected, dilute 2.5µg (2.5µl) of Arrest-In into 50µl (total volume) serum free medium into a separate microfuge tube.

c. Add the diluted DNA (step a) to the diluted Arrest-In reagent (step b), mix rapidly then incubate for 20 minutes at room temperature.

*This will give a 1:5 DNA:Arrest-In ratio which is recommended for optimal transfection into HEK293T cells. Your total volume will be 100µl at this stage.*

d. Set up all desired experiments and controls in a similar fashion as outlined in Table 5. It is also advisable to set up an Arrest-In only control.

**Table 5.** Quantities of DNA for transfection experiments

<table>
<thead>
<tr>
<th>Type of transfection experiment</th>
<th>shRNA Plasmid DNA (ng)</th>
<th>Reporter* (ng)</th>
<th>Carrier DNA** (ng)</th>
<th>Serum free medium (final volume in µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shRNA plasmid DNA</td>
<td>500 – hairpin to gene of interest</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Transfection efficiency</td>
<td>0</td>
<td>500</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Knockdown efficiency of reporter</td>
<td>450-500 – hairpin to reporter</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Control for knockdown efficiency</td>
<td>0</td>
<td>50</td>
<td>450-500</td>
<td>50</td>
</tr>
<tr>
<td>Non-silencing control</td>
<td>500 – control hairpin</td>
<td>0</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

*It is not necessary to transfect a reporter into cells if you are using a construct which already has a reporter for convenient estimation of transfection efficiency. Recommended reporters for other vectors include GFP, luciferase, and/or β-gal (X-gal staining and/or ONPG assays).

**Carrier DNA is required to increase the total DNA quantity for the formation of adequate DNA/Arrest-In complexes. Recommended carriers are pUC19 or pBluescript plasmids.*
3. Aspirate the growth medium from the cells. Add an additional 150µl of serum free medium to each of the tubes containing transfection complexes and mix gently. Add the 250µl DNA/Arrest-In™ complex mixture to the cells and incubate for 3-6 hours in a CO2 incubator at 37°C.

   *Your total volume will be 250µl at this stage.*

4. Following the 3-6 hour incubation, add an equal volume of growth medium (250µl) 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 – Arrest-In has displayed low toxicity in the cell lines tested, therefore removal of transfection reagent is not required for many cell lines. In our hands 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 reduction in gene or reporter activity by quantitative/real-time RT-PCR, western blot or other appropriate functional assay; compare to untreated, reporter alone, non-silencing shRNA or other negative controls.

   *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 RT-PCR generally gives the best indication of expression knock-down. The use of western blots to determine knock-down 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 (optional), transfer the cells to medium containing puromycin for selection. It is important to wait at least 48 hours before beginning selection.

   *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 shRNA transfectants. Typically, the working concentration ranges from 1-10µg/ml. You should use the lowest concentration that kills 100% of the cells in 1-4 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/Arrest-In mixture should be added.
to cells (post 20 minute incubation for complex formation) to a total volume of 250µl
serum free medium or to a total volume of 250µl of medium with serum (no antibiotics).

**Transfection Optimization using Arrest-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 Arrest-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 V-PACKAGING LENTIVIRUS**

For packaging our lentiviral shRNA constructs, we recommend the Trans-Lentiviral™
shRNA Packaging System (TLP4614, TLP4615). The Trans-Lentiviral shRNA
Packaging System allows creation of a replication-incompetent, HIV-1-based lentivirus
which can be used to deliver and express your gene or shRNA of interest in either
dividing or non-dividing mammalian cells. The Trans-Lentiviral shRNA 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
pLKO.1™ with our Trans-Lentiviral shRNA Packaging System, please see the product
manual available at the following link:

**PROTOCOL VI-TRANSDUCTION**

**Viral Titering**

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™ cell line that is
available as part of our Trans-Lentiviral shRNA Packaging System. You can use a
standard 293T cell line as an alternative.

*Note: If you have generated a lentiviral stock of the expression control (e.g.
pPLKO.1 eGFP), we recommend titering this stock as well.*

1. The day before transduction, seed a 24 well tissue culture plate with TLA-
   HEK293T cells at 5 x 10^4 cells per well in DMEM (10% FBS, 1% pen-strep).
   *The following day, the well should be no more than 40-50% confluent.
   TLA-HEK293T (Open Biosystems catalog no. HCL4517).*

2. Make dilutions of the viral stock in a round bottom 96 well plate using Dilution
   Media (DMEM containing 0.5% FBS and 8µg/ml polybrene). Utilize the plate as
   shown in Figure 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 5-fold dilutions to reach a final dilution of 390625-fold.
   *Polybrene is a cation that is often pre-incubated with the virus particles to
give it a net positive charge, which helps counteract the negatively-
charged cell surface membrane. Polybrene – (Sequabrene™, Sigma catalog no. S-2667).

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

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

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

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

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

   *Pipette 10-15 times and discard pipette tips.*

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

   *Pipette 10-15 times and discard pipette tip.*

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

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

8. Label 24 well plate as shown in Figure 6 using one row for each virus stock to be tested.
Figure 6. Twenty four well tissue culture plate, seeded with TLA-HEK293T™ cells, used to titer the virus.

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

10. Add 225 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 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 6).

Table 6. 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>
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<tbody>
<tr>
<td>Originating (96 well plate)</td>
<td>Destination (24 well plate)</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>25µl</td>
<td>5 *</td>
</tr>
<tr>
<td>A2</td>
<td>A1</td>
<td>25µl</td>
</tr>
<tr>
<td>A3</td>
<td>A2</td>
<td>25µl</td>
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<tr>
<td>A4</td>
<td>A3</td>
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<tr>
<td>A5</td>
<td>A4</td>
<td>25µl</td>
</tr>
<tr>
<td>A6</td>
<td>A5</td>
<td>25µl</td>
</tr>
<tr>
<td>A7</td>
<td>A6</td>
<td>25µl</td>
</tr>
<tr>
<td>A8</td>
<td></td>
<td>25µl</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.

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

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

14. Culture cells for 48 hours.

15. Count the TurboGFP expressing cells or colonies of cells (Figure 7).
Count each multi-cell colony as 1 transduced cell, as the cells will be dividing over the 48 hour culture period. Figure 7 illustrates this principle of counting.

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.
55 (TurboGFP positive colonies) \times 3125 (dilution factor) \times 40 = 6.88 \times 10^6 \text{ TU/ml}

Figure 7. Examples of individual colonies

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. 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.

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 - 8 x 10⁴ cells per well in a 24 well plate in enough wells to carry out your puromycin dilutions. Incubate overnight.
2. Prepare media specifically for your cells containing a range of antibiotic, for example: 0 - 15µg/ml puromycin.
3. The next day (day 1) replace the growth media with the media containing the dilutions of the antibiotic into the appropriate wells.
4. Incubate at 37°C.
5. Approximately every 2–3 days replace with freshly prepared selective media.
6. Monitor the cells daily and observe the percentage of surviving cells. Optimum effectiveness should be reached in 1- 4 days under puromycin selection.
7. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 1–4 days from the start of antibiotic selection.

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 7).

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 - 8 x 10⁴ 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.
   Bring the total volume of liquid up so that it just covers the cells efficiently with serum-free media (See Table 7 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.

Table 7. 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>
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<tbody>
<tr>
<td>100mm</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>60mm</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>35mm</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>6 well</td>
<td>9.4</td>
<td>1</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>
3. Approximately 6-8 hours post-transduction, add an additional 1ml of full media (serum plus pen-strep if you are using it) to your cells and incubate overnight.

   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 replace the current full growth media with full growth media containing puromycin (if required) into the appropriate wells. If adding puromycin, use the appropriate concentration as determined based on the above “kill curve”. Incubate.

   Note: When visualizing TurboGFP expression, if less than 90% of all cells are green, it is recommended in these cases to utilize puromycin selection in order to reduce background expression of your gene of interest from untransduced cells.

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

   b. 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 shRNA. Optimum effectiveness should be reached in 3-10 days with puromycin.

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

5. Proceed to extract RNA for knockdown evaluation by quantitative RT-PCR.

### RELATED REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Vendor</th>
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<td>TRC Lentiviral eGFP shRNA positive control</td>
<td>Open Biosystems</td>
<td>RHS4459</td>
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<tr>
<td>pLKO.1 empty vector</td>
<td>Open Biosystems</td>
<td>RHS4080</td>
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<tr>
<td>Arrest-In transfection reagent 0.5ml-10mls*</td>
<td>Open Biosystems</td>
<td>ATR1740-1743</td>
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<tr>
<td>Trans-Lentiviral shRNA Packaging System</td>
<td>Open Biosystems</td>
<td>TLP4614</td>
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<tr>
<td>Trans-Lentiviral shRNA Packaging System (contains cell line)</td>
<td>Open Biosystems</td>
<td>TLP4615</td>
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FAQS
For answers to questions that are not addressed here, please email technical support at info@openbiosystems.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.

What clones are part of my collection?
A CD containing the data for this collection will be shipped with each collection. This file contains the location and accession number for each construct in the collection. This data file can be downloaded from the lentiviral pLKO.1 product page: https://www.openbiosystems.com/RNAi/shRNALibraries/

Where can I find the sequence of an individual shRNA construct?
If you are looking for the sequence of an individual shRNA construct, you can use the gene search. Just enter the catalog number or clone ID of your hairpin into the gene search, hit submit and then click on the query result. If you then click on the oligo ID (the TRC number) and then click on the word “sequence” in the details grid, the hairpin sequence is listed with the target sequence annotated.
If you are looking for the sequence of several shRNA constructs, you can access this information in the data file of the collection. This data file can be downloaded from the lentiviral pLKO.1 product page: https://www.openbiosystems.com/RNAi/shRNALibraries/

Can I use ampicillin instead of carbenicillin?
No. The TRC and the Broad Institute suggest that carbenicillin be used with the pLKO.1 vector. Constructs grown in ampicillin tend to not produce high plasmid yield.

What packaging cell line should I use for making lentivirus?
For packaging our lentiviral shRNA constructs, we recommend our 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 or shRNA of interest in either dividing or non-dividing mammalian cells. The Trans-Lentiviral Packaging System uses a replication-incompetent lentivirus based on the trans-lentiviral system developed by Kappes, et al. (2001)

Should I use a second or third generation packaging cell line for packaging TRC constructs?
The pLKO.1 vector contains a chimeric 5’ LTR, so this vector can be packaged using either second or third generation packaging systems, however 2nd generation packaging is recommended and will result in higher titers than 3rd generation. The Broad Institute and the TRC recommend and use 2nd generation packaging to make viral particles.

What restriction sites were used to clone the hairpins clone into the pLKO.1 vector?
The hairpins were cloned in at AgeI and EcoRI, but the EcoRI site is usually destroyed upon ligation.
What is the sequencing primer for the pLKO.1 vector?
The pLKO.1 sequencing primer is:
5' AAACCCAGGGCTGCCTTGAAAG 3' 1540 R

Using this primer the hairpin will show up somewhere in the frame of 180-260bp into the read. Notice it is reading in the reverse orientation (Figure 8).

![Figure 8. TRC sequencing primer](image)

**TROUBLESHOOTING**

For help with transfection or transduction of your retroviral constructs, please email technical support at info@openbiosystems.com with the answers to the questions below, your sales order or purchase order number and the catalog number or clone ID of the construct with which you are having trouble.

1. Are you using direct transfection or transduction into your cell line?
2. What did the uncut and restriction digested DNA look like on a gel?
3. What was the transfection efficiency if you used direct transfection? What transfection reagent was used?
4. Were positive and negative knockdown controls used (i.e. the empty vector or the eGFP shRNA positive control)?
5. What were the results of the controlled experiments?
6. How was knockdown measured (i.e. real-time RT-PCR or western blot)?
7. What is the abundance and the half-life of the protein? Does the protein have many isoforms?
8. What packaging cell line was used if you are using infection rather than transfection?
9. What was your viral titer?
10. What was your MOI?
11. Did you maintain the cells on puromycin after transfection or transduction?
12. How much time elapsed from transfection/transduction to puromycin 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 of your DNA using 260nm 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 Arrest-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 Arrest-In seems to be toxic to a particular cell line, try reducing the DNA:Arrest-In ratio.

SUGGESTED READING:


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