MGC premier Lentiviral ORF Expression Library

Catalog number: TOH7500
Format: Glycerol stock, 96-well plate format

This manual provides information for the storage, propagation, transfection, viral packaging, and transduction of the MGC premier Lentiviral ORF Expression Library in the **pLX304 lentiviral vector**. Appendix 1 contains the pLX304 vector map and Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

Section 1: Introduction

The MGC premier human lentiviral ORF collection was developed through the collaborative efforts of Dana-Farber Cancer Institute, The Broad Institute and the Center for Cancer Systems Biology (CCSB). The collection, which includes over 13,000 ORFs that represent approximately 11,373 genes, provides the most fully sequenced and annotated version of the human ORFeome available. For added convenience the lentiviral ORF expression vector was created to enable expression of a protein of interest with a V5 fusion tag for western blot detection, purification, co-immunoprecipitation, protein localization and FACS analysis.

Deliverable

The MGC premier Lentiviral ORF Expression Library is delivered as 96-well glycerol stock plates (with lids) – 169 plates total. Plates are sealed with aluminum sealing tape and are shipped on dry ice for next day delivery. The plates should be stored at -80C upon arrival.

QC of library

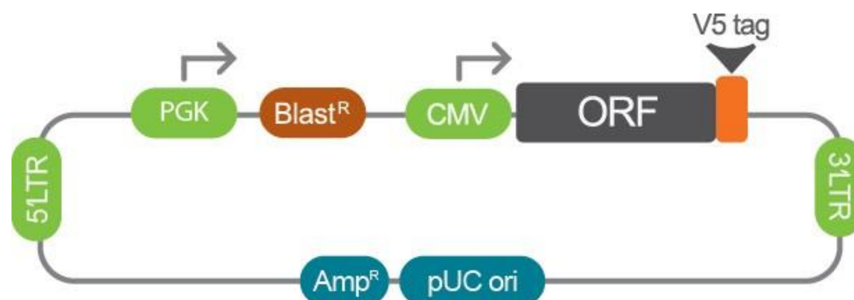
For information on the QC of the creation of the lentiviral ORF expression library, please visit:
https://www.broadinstitute.org/scientific-community/science/platforms/gpp/horfeome-v81-library
When making replica copies of the ORF library plates, Transomic ensures successful growth of each clone by comparing the growth results of each replica plate copy to the same master stock plate. To pass QC, each replica plate should have the same growth pattern as the master stock plate.

Please note that the collection contains a small percentage of clones that have been found to contain a mutation within the open reading frame and contains clones that have not been fully sequenced. These clones may prove to be useful in many experiments and are clearly marked in the data file for the collection.

Section 2: pLX304 vector information

The **pLX304 ORF expression vector** allows the stable delivery of the ORF into host cells via a replication-incompetent lentivirus.

- Ampicillin resistance for bacterial selection
- Transduction of primary and non-dividing cells
- V5 tag for additional functionality
- Blasticidin resistance for enrichment of transduced cells



5’ LTR	Hybrid Long Terminal Repeat
PGK	Drives expression of selection marker
BlastR	Blasticidin resistance marker
CMV	Drives expression of ORF
ORF	Open Reading Frame
V5 tag	Epitope tag
3’ LTR	Long Terminal Repeat

Figure 1. Schematic depicting elements of the pLX304 vector expressing the V5 tagged ORF. The vector elements table describes the utility of the various elements shown. See Appendix 1 for a more detailed vector map.

Section 3: Replication protocols

Materials

LB-Lennox Broth (low salt)	VWR 10128-266
Glycerol	VWR EM-4760
Carbenicillin (or Ampicillin)*	VWR 97063-144
96-well plates	VWR 62407-174
Aluminum seals	VWR 29445-082
Disposable replicators	Scinomix SCI-4010-OS

Individual clone propagation

Cultures should be propagated in LB broth with **ampicillin** (100 µg/ml) at 37°C for 18 hours or until the culture appears turbid. A 4 ml starter culture can be inoculated using 5 µl of the glycerol stock provided. Once turbid, place 920 µl of culture into a polypropylene tube and add 80 µl sterile glycerol (8% glycerol). Mix well and store at –80˚C. Glycerol stocks kept at -80°C are stable indefinitely if freeze/thaw cycles are minimized.
It is a good practice to streak isolate and quality control test the plasmids by restriction digest.

Replication of plates

Prepare target plates:

- Dispense ~200 µl of LB-Lennox media supplemented with 8% glycerol and 100 µg/ml ampicillin. If a lower-volume 96-well plate is substituted, then fill each well ~50% with media. Glycerol can be omitted from the media if you are culturing for a plasmid DNA extraction.

Prepare source plates

1. Remove foil seals while the source plates are still frozen. This minimizes cross-contamination.
2. Wipe any condensation underneath the lid with a paper wipe dampened with ethanol.
3. Thaw the source plates with the lid on.

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 hours or until even growth is observed in all wells.

Minimize thawed condition of plates where possible. Always store plates at -80°C. It is recommended that an archival copy is made as soon as possible. Glycerol stocks kept at -80°C are stable indefinitely if freeze/thaw cycles are kept to a minimum.

Section 4: Plasmid preparation

For transfection and transduction experiments, the **pLX304 lentiviral vector** plasmid DNA will first have to be extracted from the bacterial cells. Cultures should be grown in LB broth with ampicillin or carbenicillin (100 µg/ml) at 37°C* overnight or until the culture appears turbid. A 4 ml starter culture can be inoculated using 5 µl of the glycerol stock provided. Either a standard plasmid mini-preparation or one that yields endotoxin free DNA can be used. When isolating plasmid DNA for virus production using endotoxin free kit will generally yield higher viral titers.
***Note: The temperature for propagation is 30°C while the temperature for plasmid preparation is 37°C**

Section 5: Selection kill curve

The **pLX304 lentiviral vector** has a blasticidin resistance marker for selection in mammalian cells. To establish stable cell lines, once transfection/transduction has occurred, the cells can be treated to select for stable integrants. Since cell lines differ in their sensitivity to blasticidin the optimal concentration of blasticidin (pre-transduction) should be determined. In the following protocol the lowest concentration of blasticidin that provides adequate selection is determined for the experimental cell line.

Required materials

- Complete media for experimental cell line

- Blasticidin (1.25 µg/ml stock solution)
- 24-well tissue culture plate

Equipment

- Automatic pipette/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. Plate 5 x 10^4 cells per well in 11 wells of a 24-well tissue culture plate using media without blasticidin.
2. Prepare blasticidin dilutions in culture media for antibiotic titration as shown in **Table 1**. Use a blasticidin stock solution of 1.25 µg/µl.

Table 1. Dilutions and volumes required for establishing optimal blasticidin concentration

Volume of Blasticidin Stock Solution Added (µl)	Total Volume of Media plus Antibiotic per 24 Well (µl)	Final Concentration (µg/ml)
0	500	0
0.2	500	0.5
0.4	500	1
0.6	500	1.5
0.8	500	2
1	500	2.5
1.2	500	3
1.6	500	4
2	500	5
3	500	7.5
4	500	10

3. Begin antibiotic selection the following day by replacing antibiotic free media with media containing the appropriate concentrations of blasticidin.
4. Incubate cells with 5% CO_2 at 37°C or use conditions normal for your target cells.
5. Check cells daily to estimate rate of cell death.
6. Replenish the media containing the appropriate concentrations of blasticidin every 2 days for 6 days.
   Note: The optimal blasticidin concentration will kill the cells rapidly (2 - 4 days).

Section 6: Transfection

Use the following procedure to transfect plasmid DNA into mammalian cells in a 24-well format. For other plate formats, scale up or down the amounts of DNA and transfection reagent proportionally to the total transfection volume **(Table 2).**

Adherent cells: One day prior to transfection, plate cells in 500 µl of growth medium without antibiotics so that cells will be 70–95% confluent at the time of transfection. The number of cells to plate will vary based on the doubling time.

Suspension cells: On the same day of transfection just prior to preparing transfection complex plate 160,000 cells/well in 500 µl of growth medium without antibiotics.

Materials

- 24-well tissue culture plates
- Transfection Reagent (Examples: Lipofectamine®, Fugene®)
- Cell culture complete medium for maintenance and passaging of experimental cell line (including serum and supplements)
- Antibiotic-free complete medium for maintenance and passaging of experimental cell line (including serum or supplements) without antibiotics such as pen-strep. (i.e. Opti-MEM® I-Gibco Catalog # 51985034)
- Blasticidin S HCl antibiotic (Life Technologies, Catalog# A11139-03)
- Sterile 1.5 ml microfuge tubes

Equipment

- Automatic pipette/Pipette-aid (for tissue culture)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Transfection complex preparation (Figure 2):

Volumes and amounts are for each well to be transfected.

1. **Plasmid DNA preparation**: Dilute 0.5 µg of plasmid DNA in a microfuge tube containing Opti-MEM® I Reduced Serum Media* up to a total volume of 25 µl.
2. **Transfection reagent preparation**: In a separate microfuge tube, add 1 µL of transfection reagent into 24 µl Opti-MEM® I Reduced Serum Media* for a total volume of 25 µl.
3. **Final transfection complex**: Transfer the diluted DNA solution to the diluted transfection reagent (total volume = 50 µl). Mix gently and incubate at room temperature for 10 minutes.

Adding transfection complex to wells:

1. Add the 50 µl of transfection complex to each well containing cells and medium.
2. Incubate cells at 37°C in a CO_2 incubator for 24-48 hours.
3. After 24-48 hours of incubation, assay cells for gene activity.

** Serum-free DMEM medium can also be used.*

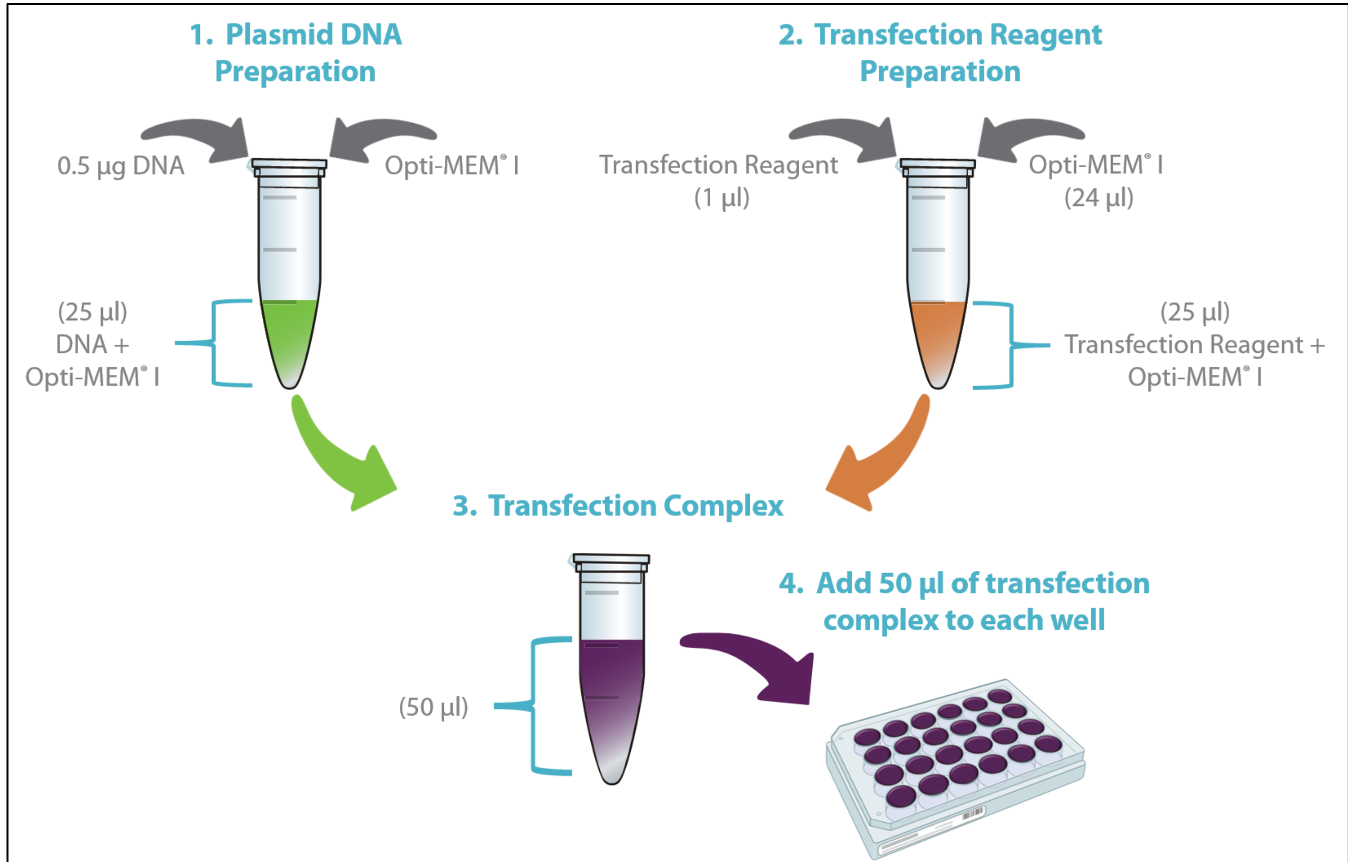


Figure 2: Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate, multiply all volumes and DNA amount by 24.

Table 2: Suggested amounts of DNA, medium and transfection reagent for transfection of plasmid DNA into adherent and suspension cells.

Tissue Culture Plates	Surface Area per Well (cm^2)	µl Plating Medium per Well	µg Plasmid DNA per Well	µl Transfection Reagent per Well	µl Transfection Complex per Well†
6- well	9	2000	2 (in 100 µl Opti-MEM® I)	4 (in 100 µl Opti-MEM® I)	200
12-well	4	1000	1 (in 50 µl Opti-MEM® I)	2 (in 50 µl Opti-MEM® I)	100
24-well	2	500	0.5 (in 25µl Opti-MEM® I)	1 (in 25µl Opti-MEM® I)	50
96-well	0.3	200	0.1 (in 10µl Opti-MEM® I)	0.2 (in 10µl Opti-MEM® I)	10-20

† Total volume of the transfection complex is made up of equal parts of DNA solution and transfection reagent.

Transfection Optimization:

It is important to optimize transfection conditions to obtain the highest transfection efficiency with lowest toxicity for various cell types. The optimal ratio of transfection reagent to DNA is relatively consistent across many cell types. For further optimization try the following steps in order.

1. Use the recommended ratio of DNA to transfection reagent (at 1 µg DNA:2 µl transfection reagent) but vary the volume.
    a. Start with a range of volumes that cover +20% to -20%.
       For example, in a 24-well plate a range of 40 µl to 60 µl of transfection complex would be added to the well. (The plating media would remain the same.)
2. If further optimization is needed, transfection efficiency and cytotoxicity may be altered by adjusting the ratio of DNA (µg) to transfection reagent (µl). A range of ratios from 1:1.5 to 1:2.5 is recommended.

Note: If transfection conditions result in unacceptable cytotoxicity in a particular cell line the following modifications are recommended:

1. Decrease the volume of transfection complex that is added to each well.
2. Higher transfection efficiencies are normally achieved if the transfection medium is not removed. However, if toxicity is a problem, aspirate the transfection complex after 6 hours of transfection and replace with fresh growth medium.
3. Increase the cell density in your transfection.
4. Assay cells for gene activity 24 hours following the addition of transfection complex to cells.

Blasticidin selection of transfected cells

Use blasticidin selection to reduce background from untransfected cells. Refer to the protocol for the blasticidin kill curve in section 5 to determine the optimal concentration for each cell line.

1. Begin the antibiotic selection by replacing the medium with complete medium supplemented with blasticidin.
2. Replace the selective media every 2-3 days. Monitor the cells daily and observe the percentage of surviving cells.
    a. All untransfected cells should be gone within 3-5 days.
3. Collect samples for assay.

If selecting stably transfected cells, continue to replace the media containing blasticidin. Observe the cells for approximately 7 days until you see single colonies surviving the selection. Colonies can be isolated and expanded for analysis.

Section 7: Packaging Lentiviral Particles

Some cell lines are resistant to transfection. Lentiviral particles offer an alternative delivery method. The **pLX304 lentiviral vector** can be packaged into lentiviral particles for efficient delivery into target cell lines.

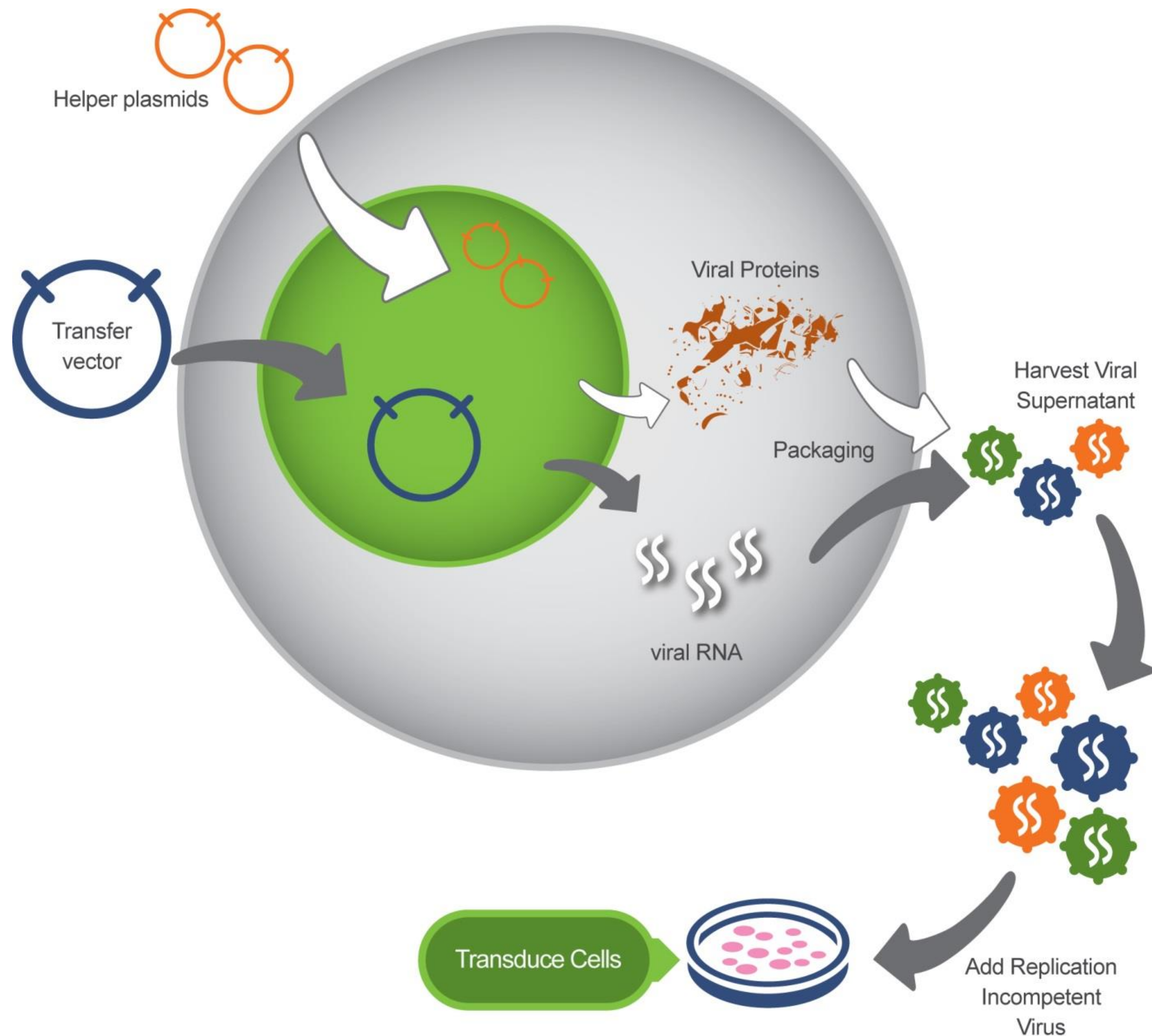


Figure 3: Schematic depicting lentiviral packaging of lentiviral vectors

When packaging lentivirus, the genetic elements required for assembly of replication incompetent viral particles are transfected into the cell in trans. The lentiviral transfer vector (pLX304) is co-transfected with the desired packaging vectors (helper plasmids) encoding the *env, gag* and *pol* protein into a packaging cell line. *gag*, *pol* and *env* provide the proteins necessary for viral assembly and maturation. The transfer vector contains sequences that will be packaged as the viral genome and code for the ORF and selection cassette that will integrate into the targeted cell's genome. Viral particles are released from the packaging cell and can be harvested from the supernatant of the packaging cell. The resulting viral supernatant can be concentrated or applied directly to the targeted cell line.

Materials

- pCMV-dR8.2 Packaging Plasmid (Addgene, Plasmid 8455)
- pCMV-VSVG Envelope Plasmid (Addgene, Plasmid 8454)
- 6-well tissue culture plate (For large ORF insert sequences, consider scaling up to a 10cm plate)
- HEK293T cells
- Complete cell culture medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine, and 1X Pen-Strep)
- Antibiotic-free complete medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine)
- Transfection Reagent
- OPTI-MEM® I + GlutaMAX Reduced Serum Media (Gibco, Catalog # 51985-034)
- Sterile 1.5 ml microfuge tubes

Equipment

- Automatic pipette/Pipette-aid (for tissue culture)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. Plate your target cells and HEK293T cells 18-24 hours prior to transduction in a 6-well plate. Plate at a density of 800,000 to 1,000,000 cells per well in 2 ml complete cell culture medium. It is important to seed enough cells so that the cell confluency ranges between 70 and 80% at the time of transfection.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Two hours prior to transfection, remove the culture media and replace with 2 ml fresh, antibiotic-free culture medium.
4. Preparation of **pLX304** plasmids and lentiviral vector packaging mix for transfection (note, all plasmids are re-suspended in dH_2O):
    - Transfer vector (**pLX304**) – dilute plasmid to 0.2 μg/μl
    - Lentiviral packaging mix (0.5 μg/μl):
        - 100 μl pCMV-dR8.2 (0.5 μg/μl)
        - 50 μl pCMV-VSVG (0.5 μg/μl)
5. Just prior to transfection, allow transfection reagent and OPTI-MEM® I to come to room temperature.
6. Plasmid DNA preparation:
    - Add 5 μl of **pLX304** (1.0 μg) and 3 μl lentiviral packaging mix (1.5 μg) in a sterile microfuge tube containing OPTI-MEM© I Reduced Serum Media to a total volume of 100 μl.
7. Transfection reagent preparation: In a separate microfuge tube, add 5 μl of transfection reagent into 95.0 μl OPTI-MEM© I Reduced Serum Media for a total volume of 100 μl.
8. Final transfection complex: Transfer the diluted DNA solution to the diluted transfection reagent (total volume = 200 μl. Mix gently and incubate at room temperature for 10 minutes.
9. Add the 200 μl of transfection complex to each well containing HEK293T cells and medium.
10. Incubate cells at 37°C in a CO_2 incubator.
11. Collect viral particles (supernatant) 48-60 hours post-transfection.
12. Clarify supernatant by low-speed centrifugation (800xg) for 10 minutes using a tabletop centrifuge.

Aliquot supernatant into sterile cryovials and store at -80°C. *Note: 50 µl aliquots will be used in the functional titering protocol. They should be stored at -80°C overnight prior to titering to reflect any loss of function due to freeze/thaw cycle that will occur for the transduction aliquots. Freshly harvested viral particles from well-transfected cells should have a titer of approximately 1-5 x 10^6 TU/ml when measured on NIH-3T3 or HEK293T cells.*

Section 8: Functional titer and transduction optimization

The number of viral particles used and the transduction efficiency will determine the average number of lentiviral integrations into the target cell genome. The following protocol is designed to evaluate functional titer of the virus produced in the previous section. Antibiotic selection may be used to remove untransduced cells. A kill curve should be performed as described in Section 5 in this product manual.

Increasing transduction efficiency:
Optimizing transduction conditions can extend the utility of viral particles and limit cell toxicity. Several variables influence transduction efficiency including components of the media, duration of transduction, cell type, cell health and plating density. It is possible to optimize many of these variables prior to the experiment.

- Serum is a known inhibitor of transduction and should be minimized (0 - 2%) in transduction media. For cells sensitive to low serum conditions either reduce the transduction time in low serum media or increase the transduction time in complete media.
- Transduction volume should be kept to a minimum. Media should barely cover cells.
- Extending transduction incubation times may increase efficiency. However, it may be necessary to increase the volume of media applied to the cells for transduction to limit the effects of evaporation.
- Hexadimethrine bromide (Polybrene) is a cationic lipid known to enhance viral particle binding to the surface of many cell types. A range of concentration (0 - 10 µg/ml) should be tested to determine the highest transduction efficiency that can be achieved with minimal cell toxicity.
- Cell density may influence transduction efficiency. Plate cells at a range of densities to determine its effect on your cell line. Rapidly dividing cells are often transduced more efficiently.

Determining Functional Titer

Functional titer must be determined using the experimental cell line to ensure optimal transduction. The functional titer is the number of viral particles, or transducing units (TU), able to transduce the target cell line per volume and is measured in TU/ml. Cell type, media components and viral production efficiency influence functional titer. It should therefore be calculated for every batch of virus produced and every cell line.

Once a baseline titer is known, this protocol can be used to further optimize transduction efficiency. To do so, follow this procedure and alter variables known to influence transduction efficiency.

- The following protocol evaluates titer by manually counting positive colonies.
- If the packaging protocol was followed in Section 7, use the titering aliquots made to determine the titer.

- Transduction optimization should be done with empty vector control viral particles.
- HEK293T cells are readily transduced under standard conditions and are included in the protocol as a positive control for transduction.

Required Materials

- Experimental cells and HEK293T cells
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles
- Sterile Microcentrifuge tubes
- Polybrene
- Appropriate antibiotic for selection

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Titering Protocol

The following protocol represents the standard procedure followed for determining functional titers in your target cell lines and HEK293T (positive control) cells. Optimal cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line.

1. Plate your target cells and HEK293T cells 18-24 hours prior to transduction in a 24 well plate. Plate at a density of 7×10^4 cells per well in 12 wells for each cell line with complete media (see **Figure 4**). Incubate overnight with 5% CO_2 at 37°C. It is important to seed enough cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in **Table 3** and **Figure 4**.
   a. Serial dilutions can be set up in a sterile 96-well plate or in sterile micro centrifuge tubes. The number of wells or tubes needed depends on the expected titer of the viral particles (generally 5-8 wells/tubes). The higher the expected titer, the more wells/tubes needed for the dilutions.
   b. Make Dilution Media by taking serum-free cell culture media and adding Polybrene to a final concentration of 5-8 µg/ml.
   c. Add 80 µl of Dilution Media to Tube/Well 1 and then 160 µl of Dilution Media to each remaining tube (Tubes/Wells 2-5).
   d. Add 20 µl of viral particles to Tube/Well 1 and mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
   e. Transfer 40 µl from Tube/Well 1 to Tube/Well 2. Mix well and discard the tip.
   f. Transfer 40 µl from tube 2 to tube 3. Mix well and discard the tip.
   g. Repeat the procedure for the remaining tubes.
   h. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.

4. Add 200 µl of culture media containing 1% serum to each well containing cells.
5. Add 25 µl from each viral dilution to two wells for each cell line (225 µl final volume) for a total of 10 wells per cell line. The remaining 4 wells (without viral particles) should be evaluated as negative controls.
6. Rock plate gently a few times to mix.
7. Incubate overnight with 5% CO_2 at 37°C.
8. Replace the viral supernatant with complete media containing the appropriate antibiotic and allow cells to grow for 72-96 hours.
9. Colony counting: (**Note***:* Counting 50-200 colonies in a well is sufficient to provide accurate titers.)
   a. Antibiotic titering by selection and colony counting:
      i. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
      ii. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
      iii. Use a microscope to count the number of surviving colonies.
10. Use the calculation below and **Table 3** to determine functional titer.

$$(Number\ of\ colonies) \times (Dilution\ factor) \div (volume\ added\ to\ cells\ (ml)) = TU/\text{ml}$$

Example:
If the average number of colonies counted in well A5 and B5 is 70 the titer is calculated as follows:
70 colonies X 3125 ÷ 0.025 ml = 8.75 X 10^6 TU/ml

Dilution table and schematic for titration protocol

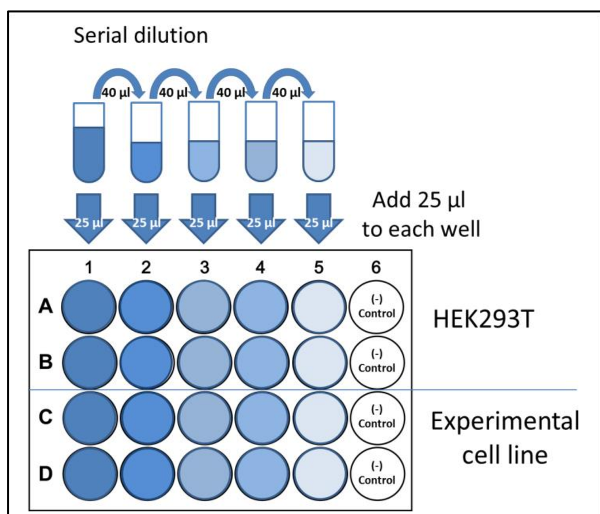


Figure 4. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3. Dilution factors for calculating viral titer

Tube/Well	Viral particles	Dilution medium	Dilution factor
1	20 µl (from virus aliquot)	80 µl	5
2	40 µl (from Tube 1)	160 µl	25
3	40 µl (from Tube 2)	160 µl	125
4	40 µl (from Tube 3)	160 µl	625
5	40 µl (from Tube 4)	160 µl	3125
6	0 µl		n/a

Example:
Typical unconcentrated virus production will yield 1-5 x 10^5 TU/ml. The expected number of colonies for a viral titer of 5 x 10^5 TU/ml would yield the following number of antibiotic resistant colonies in titering assay:

Tube	1	2	3	4	5	6
Dilution	1/5	1/5	1/5	1/5	1/5	n/a
Diluted titer TU/ml	100,000	20,000	4,000	800	160	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Antibiotic resistant colonies expected	2500	500	100	20	4	0

Counting 50-200 colonies is sufficient for an accurate measure of titer.

Section 9: Determining Multiplicity of Infection (MOI)

After the functional titer has been determined in the experimental cell line, the volume of virus required for a particular multiplicity of infection (MOI) can be calculated. The MOI is the number of transducing units per cell in a culture. The necessary MOI needed is dependent on the cell line being used and can vary widely.

Calculating volume of viral particles for a given MOI

Calculate the total number of transducing units (TU_{total}) that would be added to a well for a given MOI with the following equation:

TU_{total} = (MOI x Cell Number)/Viral titer (TU/µl)

Where:
- MOI = the desired MOI in the well (units are TU/cell)
- Cell number = number of cells in the well at the time of transduction

For example, if the experiment requires:
- MOI of 10 (highest MOI)
- Cell density of 10,000 cells per well at time of transduction
- Viral Titer is 1 x 10^7 TU/ml

Then, TU_{total} per well is calculated:
TU_{total} = (10 TU/cell) x (10,000 cells/well)] / 1 x 10^4 TU/µl = 10 µl of viral stock/well.

Therefore, the volume of viral particles with a titer of 1x10^7 TU/ml required for an MOI of 10 is 10 µl per well.

Protocol for determining optimal MOI

This protocol provides a basic outline of the transduction process. The following should be optimized prior to transduction:
- Transduction media: % Serum, Polybrene µg/ml

- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml appropriate antibiotic

Materials

- Experimental cells
- Complete media for experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles
- Sterile Microcentrifuge tubes
- Polybrene

Equipment

- Automatic pipette /Pipette-aid (for tissue culture)
- Pipette (for dilutions and handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

The following protocol represents the standard procedure followed for determining optimal MOI in HEK293T cells. Cell numbers, serum and polybrene concentrations, times, and culture conditions are likely to be different for the experimental cell line. The optimal conditions for a target cell line can be determined using the protocol for functional titer and transduction optimization (Section 8).

1. Plate cells 18-24 hours prior to transduction in a 24 well plate with complete media.   Plate at a density of cells so that the cell confluency ranges between 30 and 40% at the time of transduction.
2. Incubate overnight with 5% CO_2 at 37°C.
3. Prepare viral particles:
    a. Set up 8 sterile microcentrifuge tubes and label two tubes each with MOI. For example: 1, 2, 5, and 10.
    b. Add 50 µl of medium containing 1% serum and appropriate level of Polybrene.
    c. Add the volume of viral stock that corresponds to the MOI (use the calculation above for determining volume for the desired MOI).
    d. Bring volume in each tube up to 100 µl with medium containing 1% serum and appropriate level of Polybrene.
    e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    f. Incubate for 10 minutes at room temperature.
4. While viral particles are incubating, remove media from cells in each well.
5. Add to each well 125 µl of 1% serum media containing NO Polybrene.
6. After the 10-minute incubation, transfer all (100 µl) of virus from tubes to the corresponding wells (225 µl final volume) for a total of 8 wells (two well for each MOI). The remaining wells (without viral particles) should be evaluated as negative controls.
7. Rock plate gently a few times to distribute the viral particles across the well.
8. Incubate overnight with 5% CO_2 at 37°C (12-24 hours).
9. Replace the viral supernatant with complete media
10. Incubate cells in culture for 72-96 hours.

11. Titer by antibiotic selection and colony counting:
    a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in the previously performed "kill curve" (Section 5)
    b. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
    c. Use a microscope to count the number of surviving colonies.
    d. Calculate the titer using the number of colonies visible at the largest dilution that has colonies.

$$(Number\ of\ colonies\ ) \times (dilution\ factor) \div 0.025\ ml = \frac{TU}{ml}\ functional\ titer$$

Section 10: Transduction Guidelines & Protocols

This protocol provides a basic outline of the transduction process. The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml antibiotic

Required materials

- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with the appropriate antibiotic
- Transduction media containing viral particles (optimized for serum and Polybrene concentration)

Equipment

- Automatic pipette/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment

Protocol

Prepare cells

1. Plate cells such that they are actively dividing and 30 - 40% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction.

Transduce cells

4. Exchange media with transduction media.
   Note: Media should be serum free for maximum transduction efficiency. Alternatively, see section 8 for information on transduction optimization.
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no selection reagent).

Note: To improve transduction for non-adherent cells, cells can be moved to a round bottom tube and incubated with rotation. Rotation allows the cells and viral particles to come into contact.

Antibiotic selection

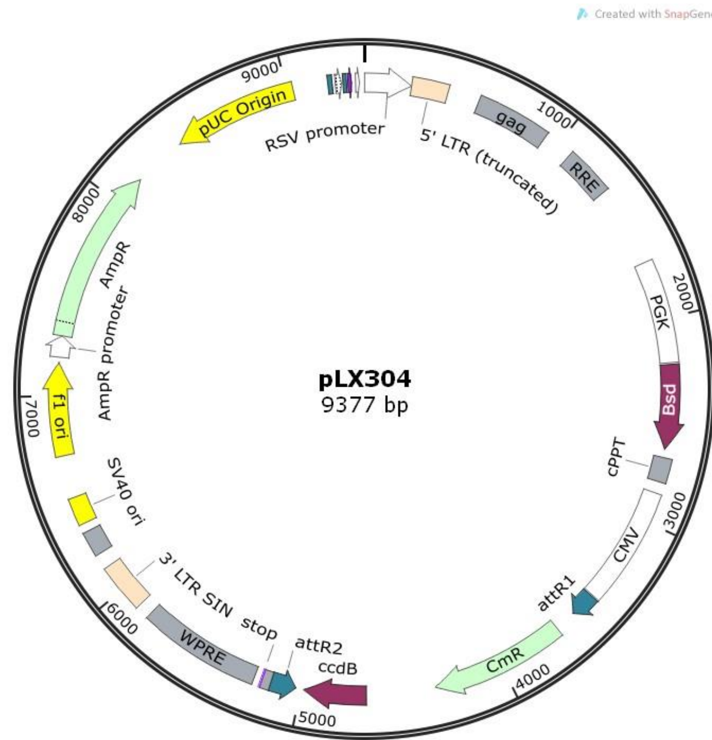
7. Allow cells to grow for 48 hours.
8. Replace media with selection media.
9. Continue feeding cells selection media until untransduced cells have been removed.

Analysis

10. Analyze expression in population. Determine cellular phenotype or harvest cell for gene expression analysis according to your experimental design.

Appendices

Appendix 1 – pLX304 vector information



Element	Start
RSV promoter	3
5' LTR	232
gag	568
RRE	1078
PGK	1714
Bsd	2226
cPPT	2681
CMV	2857
attR1	3445
CmR	3678

Element	Start
ccdB	4679
attR2	5025
V5	5151
WPRE	5234
3' LTR SIN	5893
SV40 ori	6366
f1 ori	6708
AmpR promoter	7189
AmpR	7294
pUC Origin	8415

Figure 5: Detailed map of the pLX304 vector, vector element table and sequencing primer.

The full vector sequence is available at https://www.transomic.com/cms/Product-Support/Vector-Maps-and-Sequences/CRISPR-Vector-Maps.aspx

Appendix 2 – Safety and handling of lentiviral particles

Recombinant lentivirus is considered a Biosafety Level 2 organism by the National Institutes of Health and the Center for Disease Control and Prevention. However, local health and safety regulations should be determined for each institution.

For more information on Biosafety Level 2 agents and practices, download Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (Revised December 2009) published by the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. The publication can be found here: http://www.cdc.gov/biosafety/publications/bmbl5/.

If additional measures are needed, review biosafety guidance documents such as the NIH's "Biosafety Considerations for Research with Lentiviral Vectors" which refers to "enhanced BL2 containment". More information can be found through the NIH Office of Biotechnology Activities web site (https://osp.od.nih.gov/wp-content/uploads/2014/01/Lenti_Containment_Guidance_0.pdf)

Summary of Biosafety Level 2 Practices

The following is meant to be a summary of Biosafety Level 2 practices and should not be considered comprehensive. A full account of required practices should be determined for each institute and/or department.

Standard microbiological practices

- Limit access to work area
- Post biohazard warning signs
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Review biosafety manual defining any needed waste decontamination or medical surveillance policies

Safety equipment

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is not recirculated
- Protective personal equipment includes protective laboratory coats, gloves, face protection if needed

Facilities

- Autoclave available for waste decontamination
- Chemical disinfectants available for spills

Appendix 3 – References and recommended reading

- The Broad hORFeome v8.1 Library website: https://www.broadinstitute.org/scientific-community/science/platforms/gpp/horfeome-v81-library
- The Mammalian Gene Collection: http://mgc.nci.nih.gov
- SnapGene: http://www.snapgene.com/
- NCBI: http://www.ncbi.nlm.nih.gov

Limited use licenses

This product is covered by several limited use licenses. For updated information please refer to https://www.transomic.com/cms/Company-Support/Terms.aspx

Contact Information

For more information or technical support please visit our website at www.transomic.com or contact us via email or phone.

Corporate Headquarters
Transomic Technologies Inc.
601 Genome Way, Suite 2021
Huntsville, AL 35806 USA
Phone: 866-833-0712
Fax: 256-327-9515
E-mail: support@transomic.com

Unless otherwise expressly stated on the Product or in the documentation accompanying the Product, the Product is intended for research only and is not to be used for any other purpose, including without limitation, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses.

© 2015 Transomic Technologies Inc. All rights reserved.