transOMIC technologies inc.

MGC *premier* Lentiviral V5-Tagged ORFs

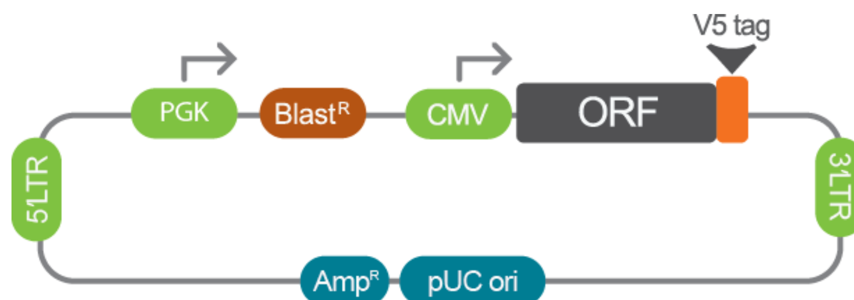
TOHV7000 (human), TOMV7000 (mouse), TORV7000 (rat)
Format: Lentiviral particle

This manual provides information for transduction with the pLX304 lentiviral vector. Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

1. pLX304 vector information

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

- 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.

2. Blasticidin selection (blasticidin kill curve)

The pLX304 lentiviral vector has a blasticidin resistance marker for selection in mammalian cells. To establish stable cell lines once transduction has occurred the cells can be placed on blasticidin 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 mg/ml stock solution)
- 24-well tissue culture plate

Equipment

- Automatic pipetter/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. Make a diluted working stock of 1 mg/ml blasticidin (blasticidin should be stored at 5-10 mg/µl stock solution).

Table 1 Dilutions and volumes required for establishing optimal blasticidin concentration

Volume of Blasticidin Stock (1 mg/ml) Solution Added (µl)	Total Volume of Media plus Antibiotic per 24 Well (µl)	Final Concentration (µg/ml)
0	500	0
1	500	2
2	500	4
3	500	6
4	500	8
5	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). This is particularly important for screens involving essential genes that may be selected against prior to the experiment.

3. 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 by selecting for resistant colonies. A kill curve should be performed as described in section 2.

Increasing transduction efficiency:
Optimizing transduction conditions can extend the utility of viral particles and limit cell toxicity. A number of 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 cells 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 for viral particle production, use the titering aliquots made in the virus production section above to determine the titer.
- HEK293T cells are readily transduced under standard conditions and are included in the protocol as a positive control for transduction.

Required materials

- 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 (Harvested or purchased)
- Microcentrifuge tubes
- Polybrene

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with GFP filter (refer to Appendix 4 for alternative methods that do not require this)

Protocol

1. Plate cells (HEK293T and experimental cell line) 24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 12 wells with complete media. Incubate for 24 hours with 5% CO_2 at 37°C.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 2 and Figure 3.
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 160 µl of serum free medium to each tube containing 5-8 µg/ml Polybrene.
    c. Add 40 µl of viral stock to the first microfuge tube.
    d. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    e. Transfer 40 µl from the first microcentrifuge tube to the second tube. Mix well and discard the tip.
    f. Repeat the procedure for the 4 remaining tubes.
    g. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 200 µl of serum free media to each well containing cells.
5. Add 25 µl from each viral dilution to two wells (225 µl final volume) for a total of 10 wells per cell line. The remaining two 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 and allow cells to grow for 48 hours.
9. Select resistant colonies using Blasticidin selection
    a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal blasticidin concentration determined in “blasticidin kill curve”
    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 functional titer using the number of colonies visible at the largest dilution that has colonies.
10. Use the calculation below and Table 2 to determine functional titer.

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

For Example:
If the average number of colonies counted in well A4 and B4 is 70 the titer is calculated as follows:
70 colonies X 625 ÷ 0.025 ml = 1.75 X 10^6 TU/ml

Dilution table and schematic for titration protocol

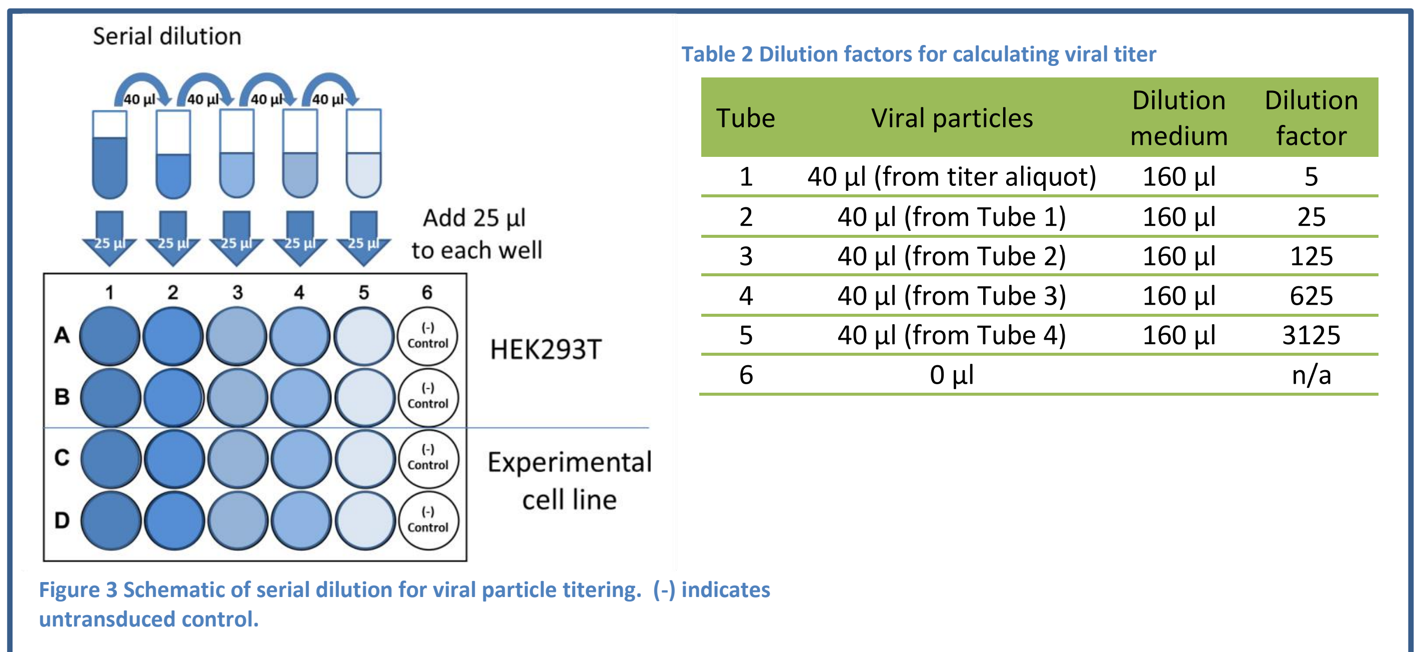


Figure 3 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 2 Dilution factors for calculating viral titer

Tube	Viral particles	Dilution medium	Dilution factor
1	40 µl (from titer aliquot)	160 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 virus production will yield 1-5 x 10^6. The expected number of fluorescent colonies for a viral titer of 1 x 10^6 would yield the following number of fluorescent 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	200,000	40,000	8,000	1,600	320	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	5,000	1,000	200	40	8	0

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

V. 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 blasticidin

Required materials

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

Equipment

- Automatic pipetter /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 60 - 70% 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 4 for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no blasticidin).

Blasticidin selection

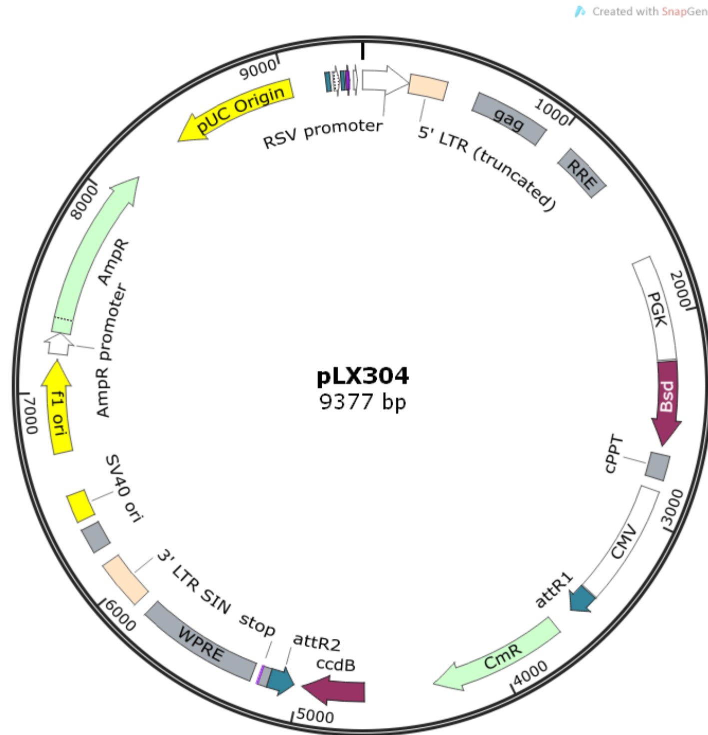
7. Allow cells to grow for 48 hours.
8. Replace media with selection media.
9. Continue feeding cells selection media until non-transduced cells have been removed.

Analysis

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

Appendices

Appendix 1 – pLX304 vector information



Element	Start	Stop
RSV promoter	3	231
5' LTR	232	412
gag	568	932
RRE	1078	1319
PGK	1714	2216
Bsd	2226	2639
cPPT	2681	2798
CMV	2857	3438
attR1	3445	3569
CmR	3678	4337

Element	Start	Stop
ccdB	4679	4984
attR2	5025	5149
V5	5151	5192
WPRE	5234	5822
3' LTR SIN	5893	6128
SV40 ori	6366	6501
f1 ori	6708	7163
AmpR promoter	7189	7293
AmpR	7294	8154
pUC Origin	8415	9030

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

The full sequence is available here.

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 (http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html)

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

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