transEDIT™ Lentiviral Paired gRNA (pCLIP-gRNA) Target Gene Sets

Bacterial Glycerol Stock

CNHS1001, CNMS1001, CNRS1001

This manual provides information for the propagation, transfection, transduction and viral packaging of the transEDIT lentiviral gRNA expression vector (pCLIP-gRNA). Cas9 Nickase must be expressed in the cells targeted with the with a pair of pCLIP-gRNA through co-transfection or the creation of a stable cell line expressing Cas9 Nickase. Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems were originally discovered as part of a prokaryotic adaptive immune system to protect against invading viruses and bacteriophages. The type II CRISPR/Cas system found in *Streptococcus pyogenes* has been well-studied, and is comprised of a CRISPR-associated (Cas9) endonuclease that complexes with two small guide RNAs, crRNA and tracrRNA, to make a double-stranded DNA break (DSB) in a sequence specific manner (Reviewed in Charpentier & Doudna, 2013). The crRNA and tracrRNA, which can be combined into a single guide RNA (gRNA), directs the Cas9 nuclease/nickase to the target sequence through base pairing between the gRNA sequence and the genomic target sequence. The target sequence consists of a 20-bp DNA sequence complementary to the gRNA, followed by trinucleotide sequence (5'-NGG-3') called the protospacer adjacent motif (PAM). The Cas9 nuclease digests both strands of the genomic DNA 3-4 nucleotides 5' of the PAM sequence. By simply introducing different guide RNA sequences, the Cas9 can be programmed to introduce site-specific DNA double-strand breaks virtually anywhere in the genome where a PAM sequence is located. The double-stranded break at the target site induces DNA repair mechanisms, such as non-homologous end joining (NHEJ) that create insertions and deletion (indels) leading to a premature stop codon, and homology-directed recombination (HR) for introducing or knocking in new sequences (Figure 1). Gene knockouts or knockins can be efficiently created in many different cell lines opening up unprecedented opportunities for targeted genome editing and cell engineering.

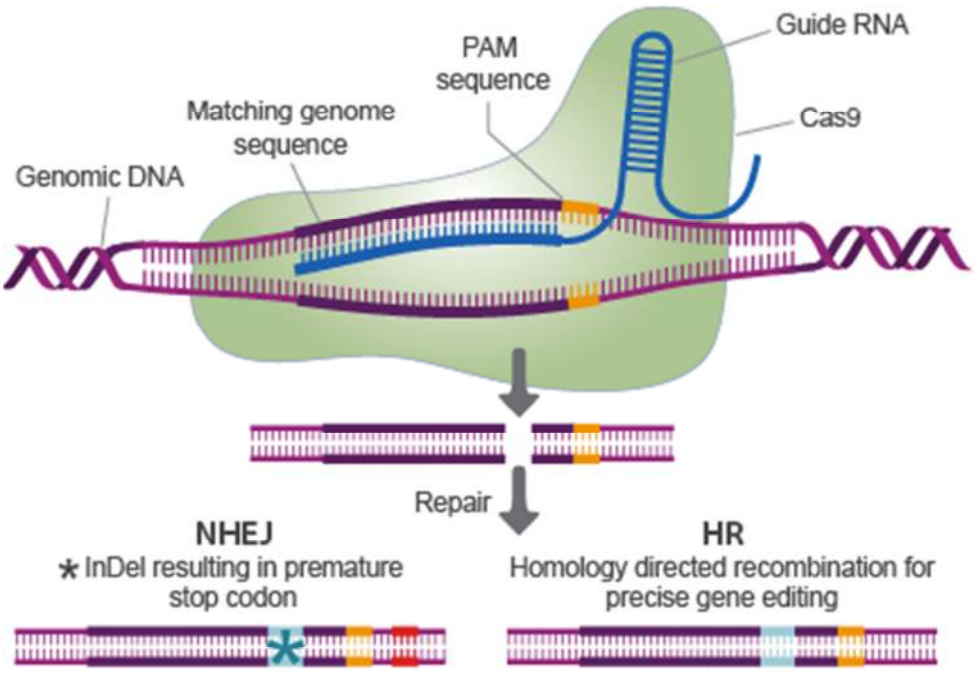


Figure 1. Schematic representation of RNA-guided double-stranded DNA cleavage by CRISPR/Cas9 using a programmable guide RNA.

2. Lentiviral gRNA plus Cas9 (pCLIP-gRNA) vector information

The pCLIP-gRNA expression vector allows transient and stable transfection; as well as the stable delivery of gRNA into host cells via a replication-incompetent lentivirus. Pairs of pCLIP-gRNA vectors are designed to be used in conjunction with a Cas9 Nickase expression vector.

- Transduction of primary and non-dividing cells
- Fluorescent marker for direct detection expression
- Puromycin or blasticidin resistance or fluorescent marker expression for enrichment of transduced cells and increased genome editing efficiency.

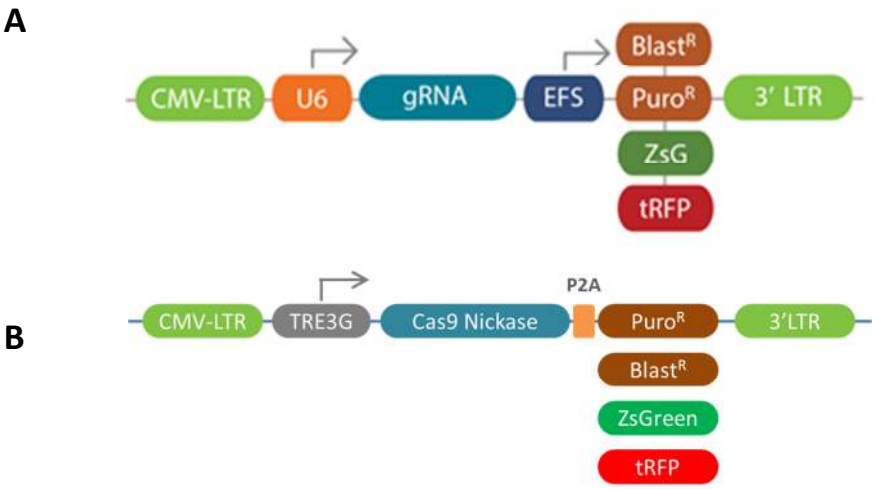


Figure 2. (A) Cartoon of the basic elements of the pCLIP-gRNA lentiviral vectors. Multiple selection markers are available. Vectors shown are pCLIP-gRNA (EFS-Blast), pCLIP-GRNA (EFS-Puro), pCLIP-gRNA (EFS-ZsGreen) and pCLIP-gRNA (EFS-tRFP). (B) Cartoon of Cas9 expression vector option for use with pCLIP-gRNA. Vectors shown are pCLIP-Cas9 Nickase (EFS-Blast), pCLIP-CAS9 Nickase (EFS-Puro), pCLIP-CAS9 Nickase (EFS-ZsGreen) and pCLIP-CAS9 Nickase (EFS-tRFP).

3. Replication protocols for glycerol stocks

Materials for individual and plate replication

LB-Lennox Broth (low salt)	VWR EM1.00547.0500
Glycerol	VWR EM-4760
Carbenicillin	VWR 97063-144
96-well plates	VWR 62407-174
Aluminum seals	VWR 29445-082
Disposable replicators	Genetix X5054

Propagate culture for storage

pCLIP-gRNA lentiviral cultures should be propagated in LB broth with ampicillin or carbenicillin (100 µg/ml) at 30°C for 30 hours or until the culture appears turbid. 2-10 ml starter cultures can be inoculated using 2 to 10 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 as long as freeze/thaw cycles are minimized.

4. Plasmid preparation

For transfection and transduction experiments the pCLIP-gRNA 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. 2-10 ml starter cultures can be inoculated using 2 to 10 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.

5. Selection kill curve

The optimal antibiotic concentration should be determined for a cell line prior to transduction or transfection. The pCLIP-gRNA and pCLIP-Cas9 Nickase vector have an option for puromycin or blasticidin selection in mammalian cells. These can be used to co-select cells for expression of both plasmids. 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 antibiotics, the optimal concentration (pre-transfection/transduction) should be determined. In the following protocol the lowest concentration that provides adequate selection is determined for the experimental cell line.

Puromycin and blasticidin have a similar range of concentration that is toxic to most cell lines. So, the same kill curve can be used for both.

Required materials

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution)
- Blasticidin (1.25 µg/µl 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 puromycin. Prepare puromycin dilutions in culture media for antibiotic titration as shown in

Table 1. Use a puromycin stock solution of 1.25 µg/µl stock solution.

Table 1 Dilutions and volumes required for establishing optimal antibiotic concentration

Volume of Puromycin 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

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

5. Co-transfection

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

The pCLIP-Cas9 Nickase Expression plasmid is co-transfected with the pCLIP-gRNA plasmid at a ratio of 4 to 1, Cas9 nickase plasmid to guide RNA plasmid. For controls, it is recommended that three additional wells be set up, (1) transfection of only the pCLIP-Cas9 Nickase plasmid without the pCLIP-gRNA plasmids, (2) co-transfection of the Cas9 Nickase plasmid with the paired pCLIP-gRNA Negative Control plasmid.

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 double time.

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

transEDIT CRISPR/Cas9 Vectors

- transEDIT gRNA vectors (pCLIP-gRNA), containing gRNA designed for your gene target of interest – target set of 3 pairs of gRNA vectors, bacterial glycerol stock
- transEDIT paired pCLIP-gRNA Negative Control, bacterial glycerol stock

Additional Materials

- 24-well tissue culture plates
- OMNIfect™ Transfection Reagent
- 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.
- Blasticidin S HCl antibiotic (Life Technologies, Cat# A11139-03)
- Puromycin Dihydrochloride (Life Technologies, Cat# A11138-03)
- Sterile 1.5 ml microfuge tubes
- Assays for assessing genome editing (e.g. Surveyor Assay)

Equipment

- Automatic pipettor/Pipette-aid (for tissue culture)
- Disposable or autoclaved tissue culture pipettes
- CO^2 cell culture incubator at 37°C
- Fluorescent microscope

Transfection complex preparation (Figure 3):

Volumes and amounts are for each well to be transfected.

1. Plasmid DNA preparation: Dilute 0.4 µg of pCLIP-Cas9 Nickase plasmid, 0.1 µg of pCLIP-gRNA(1) plasmid and 0.1 µg of pCLIP-gRNA(2) plasmid in a sterile microfuge tube containing Opti-MEM© I Reduced Serum Media* to a total volume of 25 µl.
2. OMNIfect reagent preparation: In a separate microfuge tube, add 1.2 µL of OMNIfect into 24 µl Opti-MEM® I Reduced Serum Media* for a total volume of 25.2 µl.
3. Final transfection complex: Transfer the diluted DNA solution to the diluted OMNIfect 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 48-96 hours changing media as necessary.
3. Perform assay to determine genome editing efficiency.

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

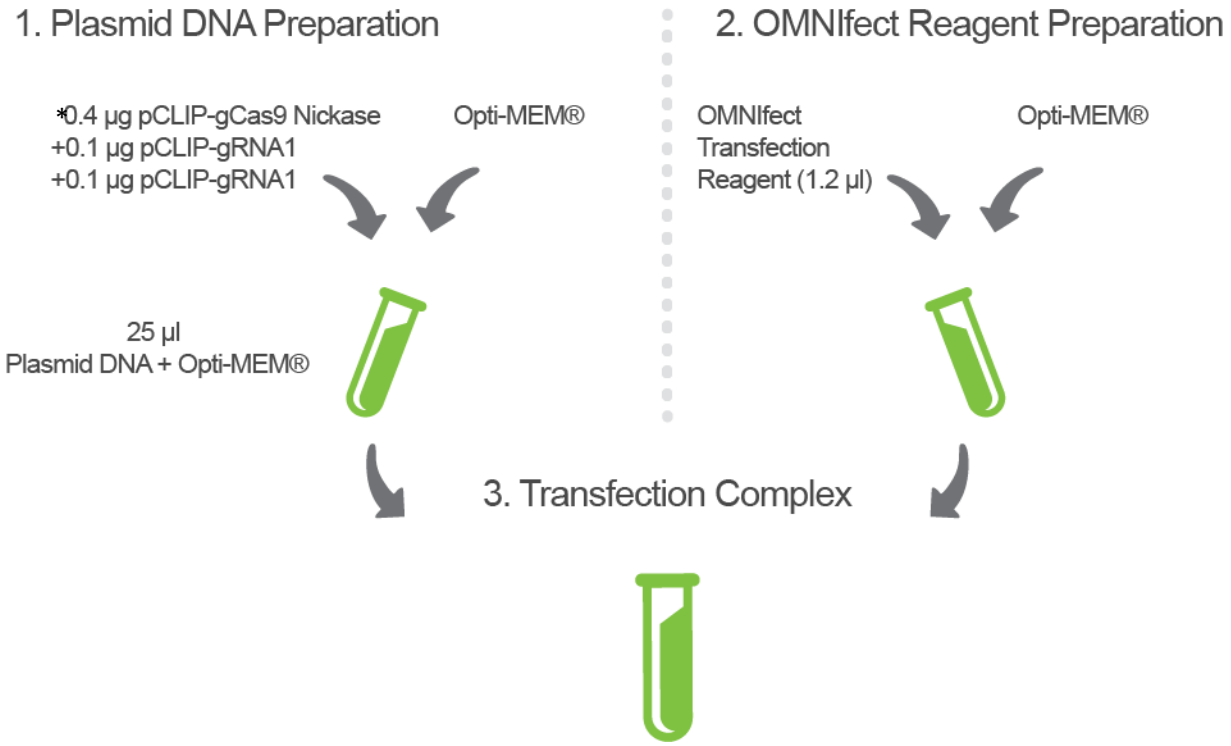


Figure 3: Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24. *0.6 µg of DNA in step one represents the 0.4 µg of pCLIP-Cas9 plasmid combined with 0.1 µg of pCLIP-gRNA1 and 0.1 µg of pCLIP-gRNA2 plasmid.

Table 2: Suggested amounts of DNA, medium and OMNIfect 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 OMNIfect per Well	µl Transfection Complex per Well†
6- well	9	2000	2.4 (in 100 µl Opti-MEM® I)	4.8 (in 100 µl Opti-MEM® I)	200
12-well	4	1000	1.2 (in 50 µl Opti-MEM® I)	2.4 (in 50 µl Opti-MEM® I)	100
24-well	2	500	0.6 (in 25µl Opti-MEM® I)	1.2 (in 25µl Opti-MEM® I)	50
96-well	0.3	200	0.11 (in 10µl Opti-MEM® I)	0.22 (in 10µl Opti-MEM® I)	10-20

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

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 OMNIfect 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:transfection reagent (at 1 µg DNA:2 µl OMNIfect), 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 OMNIfect 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.

Selection - Enrichment for increased genome editing efficiency

After 24-48 hours, transfected cells can be selected using antibiotic resistance or fluorescent protein (FP) expression. The transEDIT pCLIP-gRNA Lentiviral particles co-express several different selectable markers via a 2A "self-cleaving" peptide, including blasticidin resistance (Blast^R) and puromycin resistance (Puro^R) genes, as well as ZsGreen, and turboRFP (tRFP) FPs. Antibiotic selection ensures the removal of untransfected cells. Using FACS analysis to select for cells with highest fluorescent protein expression can further enrich for the population of cells with the highest frequency of genome editing.

Antibiotic selection:

Refer to the protocol for the puromycin or blasticidin kill curve in section 5 to determine the optimal concentration for each cell line. Puromycin and blasticidin may be used in parallel for many cell lines. If there are insufficient cells remaining following dual selection then we recommend packaging the vectors as lentiviral particles for efficient delivery and serial selection.

1. Incubate for 24-72 hours following transfection and then examine the cells microscopically for growth.
2. Begin the antibiotic selection by replacing the medium with complete medium supplemented puromycin or blasticidin.
3. 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 removed within 3-5 days.
4. Collect samples for assay.

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

Fluorescence analysis: If the vector expresses a fluorescent protein, incubate for 24-72 hours following transfection and then examine the cells microscopically for fluorescence expression. Sort the cells based on level of fluorescence and select the highest expressing population.

Single-Cell Cloning

It may be necessary to isolate clonal populations of cells so that each cell in the culture contains the desired edited genome or to select for a Cas9 expressing cell for co-transfection with paired gRNA. Clonal populations may also be required to confirm that all alleles of a particular gene within the target cell are knocked out. If cells transfected with CRISPR/Cas9 vectors co-express a FP, single-cell clones can be generated using FACS to seed single cells into a 96-well plate. If FACS is not readily available, then clonal cells can be generated manually by simply diluting the cells and plating them in a 96-well plate. Cells should be seeded at a cell number ranging from 0.5 to 2.0 cells per well in a total volume of 100 µl/well of complete culture medium. It is recommended that several 96-well plates be plated for each number of cells in the range. If using adherent cells, cultures growing out from one colony can be identified by microscopy and expanded. Suspension cells are more difficult to clonally isolate and therefore may require seeding cells at a lower cell concentration (0.1 cells per well). It is assumed that cells growing out in wells seeded with such a low cell concentration are probably derived from a single, clonal cell.

6. Packaging lentiviral particles

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

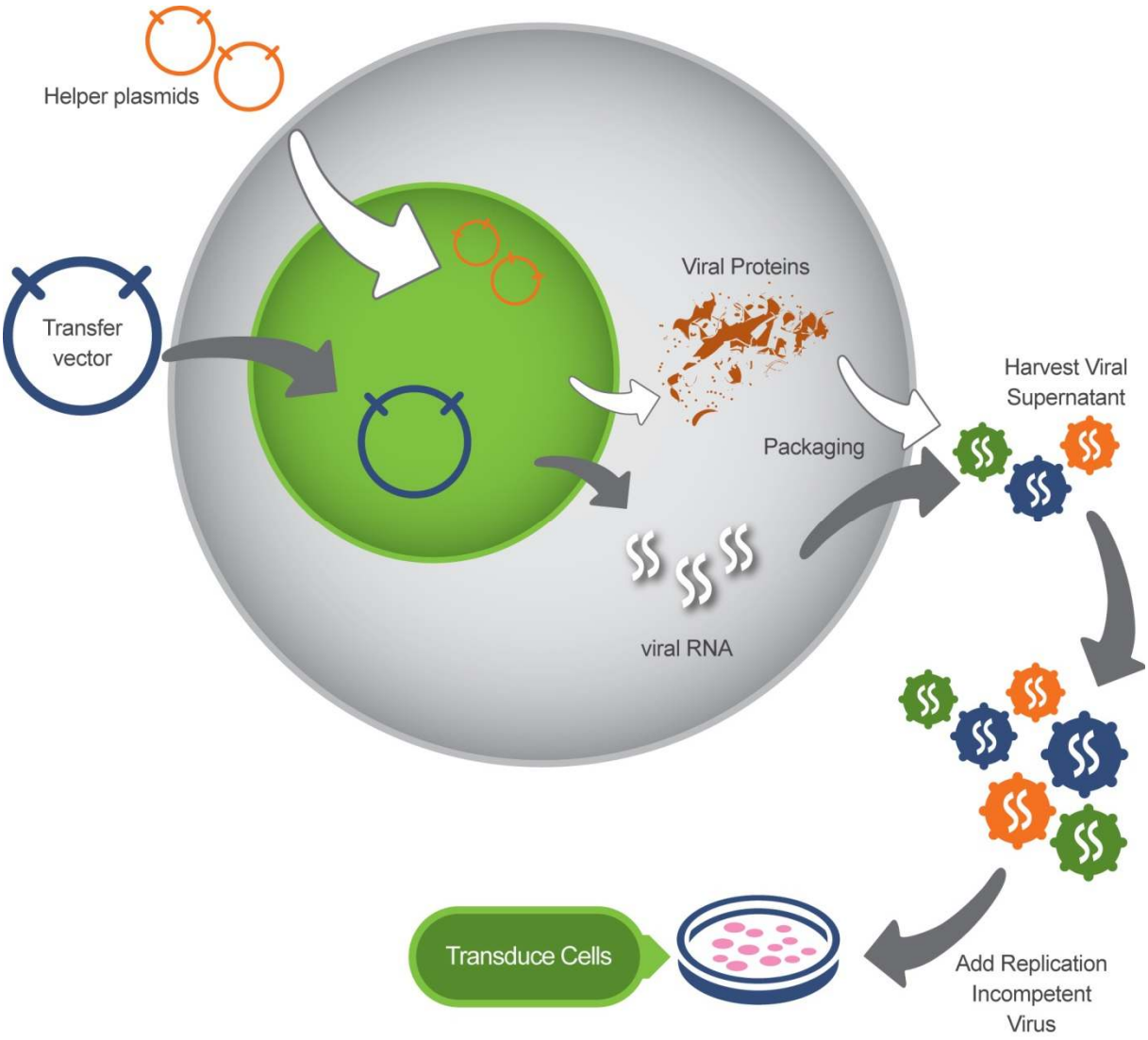


Figure 4: Schematic depicting lentiviral packaging of lentiviral vectors

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 is co-transfected with the desired packaging vectors 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 CRISPR/Cas9 components and selection cassette that will integrate into the target 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-dR8.91 Packaging Plasmid (Addgene, Plasmid 8455)
- pCMV-VSVG Envelope Plasmid (Addgene, Plasmid 8454)
- 6-well tissue culture plate
- HEK293T cells
- Complete cell culture medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine, and 1X Pen-Strep)
- Serum-free complete medium – (DMEM supplemented with 10% fetal calf serum, 1X L-Glutamine)
- Omnifect Transfection Reagent
- OPTI-Mem® I + GlutaMAX Reduced Serum Media (Gibco, Cat. # 51985-034)
- Sterile 1.5 ml microfuge tubes

Equipment

- Automatic pipettor/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 serum-free complete media. 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. Preparation of CRISPR/Cas9 plasmids and lentiviral vector packaging mix for transfection (note, all plasmids are resuspended in dH2O):
    a. Transfer vector (pCLIP-Cas9 Nickase, pCLIP-gRNA) – dilute plasmid to 0.2 μg/μl
    b. Lentiviral packaging mix (0.5 μg/ μl):
        i. 100 μl pCMV-dR8.91 (0.5 μg/μl)
        ii. 50 μl pCMV-VSVG (0.5 μg/μl)
4. Just prior to transfection, allow Omnifect and OPTI-Mem® I to come to room temperature.
5. Plasmid DNA preparation: Add 5 μl of CRISPR/Cas9 plasmid (1.0 μg) and 3 μl of 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.
6. OMNIfect™ reagent preparation: In a separate microfuge tube, add 5 μl of OMNIfect™ into 95.0 μl Opti-MEM© I Reduced Serum Media*** for a total volume of 100 μl.
7. Final transfection complex: Transfer the diluted DNA solution to the diluted OMNIfect™ reagent (total volume = 200 μl. Mix gently and incubate at room temperature for 10 minutes.
8. Add the 200 μl of transfection complex to each well containing HEK293T cells and medium.
9. Incubate cells at 37°C in a CO_2 incubator.
10. Collect viral particles (supernatant) 48-60 hours post-transfection.
11. 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: 2 x 50 μl aliquots will be used in the functional titering protocol for pCLIP-gRNA. 1 ml aliquots will be needed for titering pCLIP-Cas9 Nickase. 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.*

7. 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. Selection may be used to remove untransduced cells. A kill curve should be performed as described in section 5.

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 ZsGreen positive colonies. Alternate methods for determining titer are provided in Appendix 3.
- Transduction optimization should be done with the with Non-Targeting Control viral particles.
- 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 Appendix3 for alternative methods that do not require this)

Protocol – Titering CLIP-gRNA vectors

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. (Follow the alternate protocol when titering Cas9 expressing vectors.)
    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. Colony counting: (*Note: Counting 50-200 colonies in a well is sufficient to provide accurate titers.*)
    a. Puromycin or blasticidin 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.
    b. Fluorescent colony counting
        i. Count the number of colonies expressing ZsGreen or tRFP. A colony consisting of multiple cells should be counted as a single transduction event.
9. Use the calculation below and Table 2 to determine functional titer.
   (An alternate method for calculating titers via FACS is described in Appendix 3.)

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

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

Alternate Titering Protocol for the Cas9 Nickase

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 3 and Figure 4.
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 1.25 ml of viral supernatant to the first tube.
    c. Add 1 ml serum free medium with 5-8 µg/ml Polybrene to each of the remaining tubes.
    d. Transfer 250 µl from the tube 1 to tube 2.
    e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    f. Repeat the procedure for tubes 3-5 leaving tube 6 with no viral particles.
    g. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 250 µl from each viral dilution to two wells for a total of 10 wells per cell line. The remaining two wells (without viral particles) should be evaluated as negative controls.
5. Rock plate gently a few times to mix.
6. Incubate overnight with 5% CO_2 at 37°C.
7. Colony counting: (*Note: Counting 50-200 colonies in a well is sufficient to provide accurate titers.*)
    a. Puromycin or blasticidin 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.
    b. Fluorescent colony counting
        i. Count the number of colonies expressing ZsGreen or tRFP. A colony consisting of multiple cells should be counted as a single transduction event.
8. Use the calculation below and Table 3 to determine functional titer.
   (An alternate method for calculating titers via FACS is described in Appendix 3.

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

Standard dilution table and schematic for titration protocol

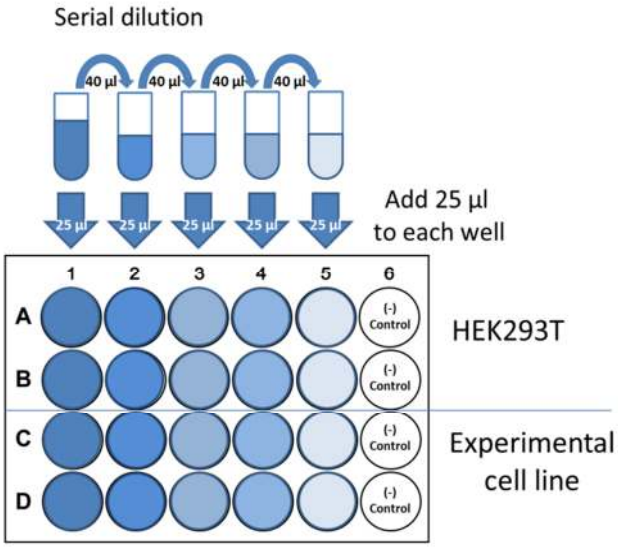


Figure 3 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

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:
Following this protocol with a viral titer of 1×10^6, column 3 of the plate (1/125 dilution) would be expected to have 200 colonies.

Alternate dilution table and schematic for titration protocol (Used for Cas9 vector)

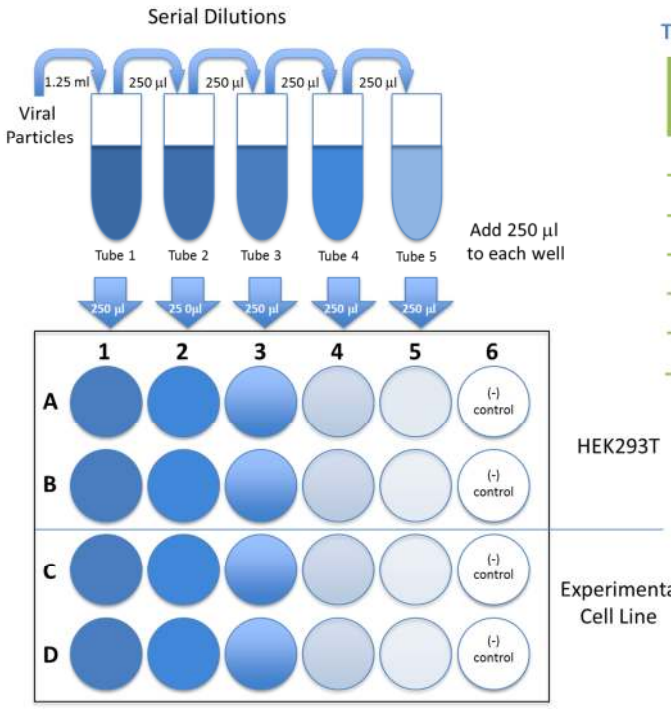


Figure 4 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3 Dilution factors for calculating viral titer

Tube	Viral particles	Dilution medium	Dilution factor
1*	1.25 ml (from titer aliquot)	0 µl	1
2	250 µl (from Tube 1)	1 ml	5
3	250 µl (from Tube 2)	1 ml	25
4	250 µl (from Tube 3)	1 ml	125
5	250 µl (from Tube 4)	1 ml	625
6	0 µl		n/a

*For Cas9 expressing vectors start with undiluted

Example:
Following this protocol with a viral titer of 1×10^4, column 3 of the plate (1/25 dilution) would be expected to have 100 colonies.

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 puromycin or 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 7 for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no selection reagent).

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

Selection - Enrichment for increased genome editing efficiency

After 24-48 hours, transduced cells can be selected using antibiotic resistance or fluorescent protein (FP) expression. The transEDIT pCLIP-gRNA Lentiviral particles co-express several different selectable markers via a 2A "self-cleaving" peptide, including blasticidin resistance (Blast^R) and puromycin resistance (Puro^R) genes, as well as ZsGreen, and turboRFP (tRFP) FPs. Antibiotic selection ensures the removal of untransduced cells. Using FACS analysis to select for cells with highest fluorescent protein expression can further enrich for the population of cells with the highest frequency of genome editing.

Antibiotic selection:

Refer to the protocol for the puromycin or blasticidin kill curve in section 5 to determine the optimal concentration for each cell line.

1. Incubate for 24-72 hours following transduction and then examine the cells microscopically for growth.
2. Begin the antibiotic selection by replacing the medium with complete medium supplemented puromycin or blasticidin.
3. Replace the selective media every 2-3 days. Monitor the cells daily and observe the percentage of surviving cells.
    a. All untransduced cells should be removed within 3-5 days.
4. Collect samples for assay.

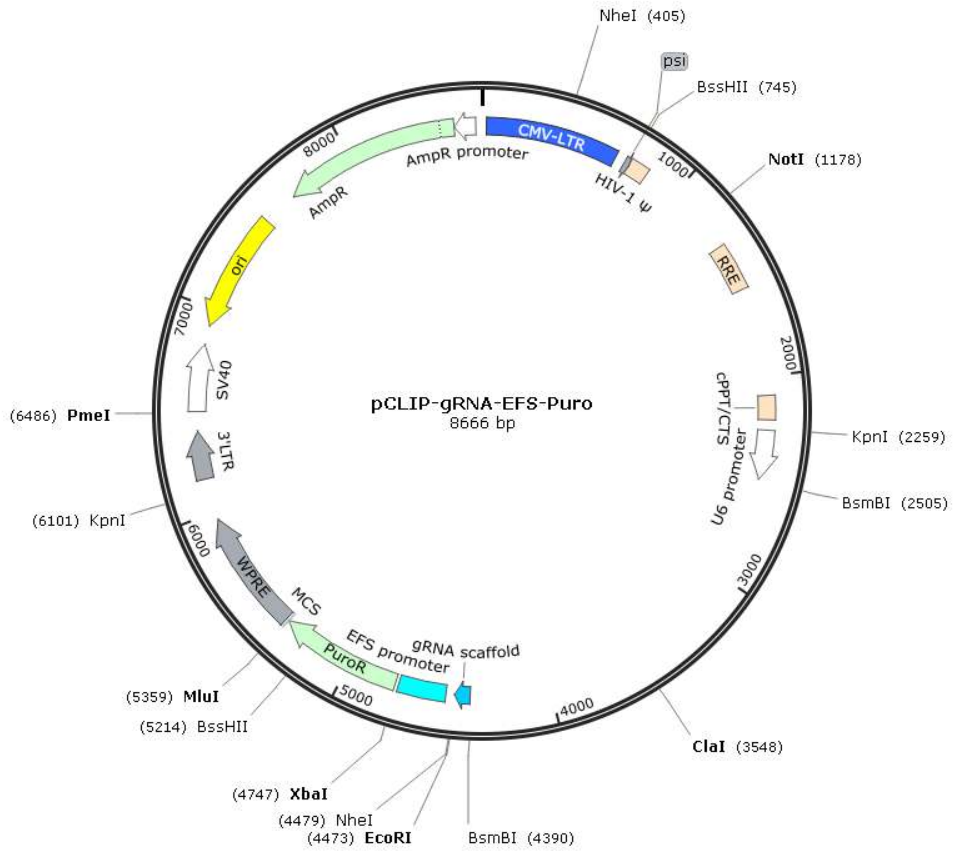
Fluorescence analysis: If the vector expresses a fluorescent protein, incubate for 24-72 hours following transduction and then examine the cells microscopically for fluorescence expression. Sort the cells based on level of fluorescence and select the highest expressing population.

Single-Cell Cloning

It may be necessary to isolate clonal populations of cells so that each cell in the culture contains the desired edited genome. Clonal populations may also be required to confirm that all alleles of a particular gene within the target cell are knocked out. If cells transduced with CRISPR/Cas9 vectors co-express a FP, single-cell clones can be generated using FACS to seed single cells into a 96-well plate. If FACS is not readily available, then clonal cells can be generated manually by simply diluting the cells and plating them in a 96-well plate. Cells should be seeded at a cell number ranging from 0.5 to 2.0 cells per well in a total volume of 100 µl/well of complete culture medium. It is recommended that several 96-well plates be plated for each number of cells in the range. If using adherent cells, cultures growing out from one colony can be identified by microscopy and expanded. Suspension cells are more difficult to clonally isolate and therefore may require seeding cells at a lower cell concentration (0.1 cells per well). It is assumed that cells growing out in wells seeded with such a low cell concentration are probably derived from a single, clonal cell.

Appendices

Appendix 1 – pCLIP-gRNA-EFS-Puro



Element	Start	Stop
CMV-LTR	23	667
psi	720	745
HIV-1 Psi	746	840
RRE	1333	1566
cPPT/CTS	2093	2210
U6 promoter	2261	2501
gRNA scaffold	4391	4466
EFS promoter	4508	4740

Element	Start	Stop
PuroR	4759	5358
MCS	5360	5365
WPRE	5372	5963
3'LTR	6168	6403
SV40	6497	6826
ori	6915	7500
AmpR	7671	8531
AmpR promoter	8532	8636

Figure 5: Detailed map of the pCLIP-gRNA-EFS-Puro vector. The pCLIP vectors includes options for additional selection markers. See the website for detailed vector information for the other vectors.

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

Appendix 3 - Alternate methods for titering

1. Puromycin or blasticidin titering by selection and colony counting:
   a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "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.

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

2. ZsGreen titering by FACS analysis
   a. When calculating the percentage of transduced cells **use the number of cells present on the day of transduction as the denominator**.
   b. Only analyze wells that have < 20% of cells transduced to ensure none of the cells have been transduced with more than one viral particle.

$$\frac{(Number\ of\ cells\ at\ transduction\ )}{(Number\ of\ ZsGreen\ positive\ cells\ in\ well)} \times (dilution\ factor) \div 0.025\ ml = \frac{TU}{ml}\ functional\ titer$$

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