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

Viral particles

CCHV1001, CCMV1001, CCRV1001

This manual provides information for the transduction of the transEDIT lentiviral gRNA expression vector (pCLIP-gRNA). Cas9 must be expressed in the cells targeted with the pCLIP-gRNA through co-transduction or the creation of a stable cell line expressing Cas9. 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 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.

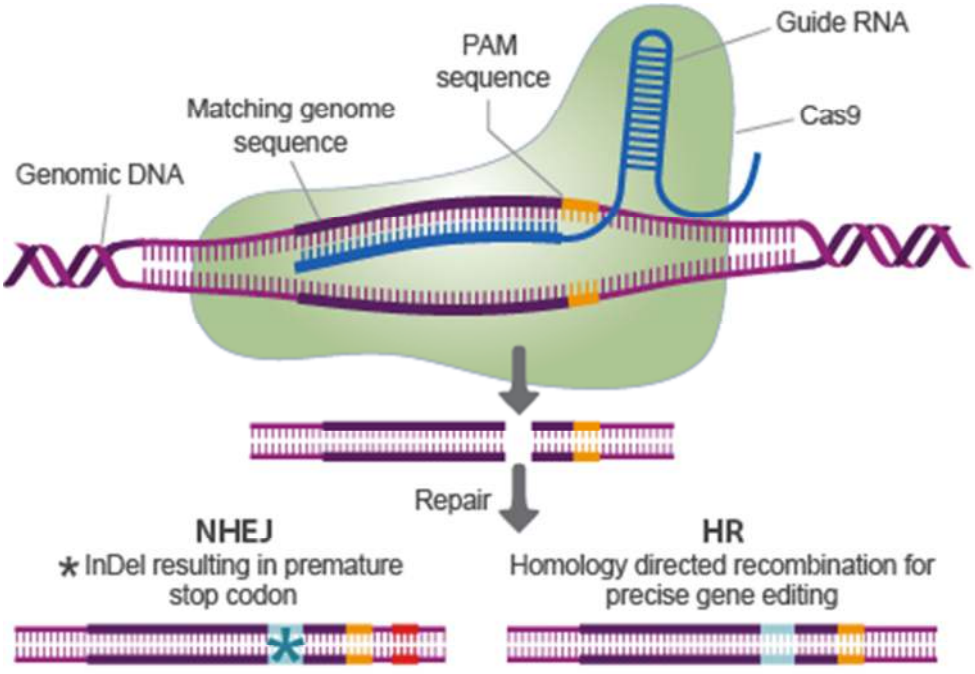

Figure 1. Schematic representation of RNA-guided double-stranded DNA cleavage by CRISPR/Cas9 using a programmable guide RNA.

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

The pCLIP-gRNA expression vector allows the stable delivery of gRNA into host cells via a replication-incompetent lentivirus. The pCLIP-gRNA vector is designed to be used in conjunction with a Cas9 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.

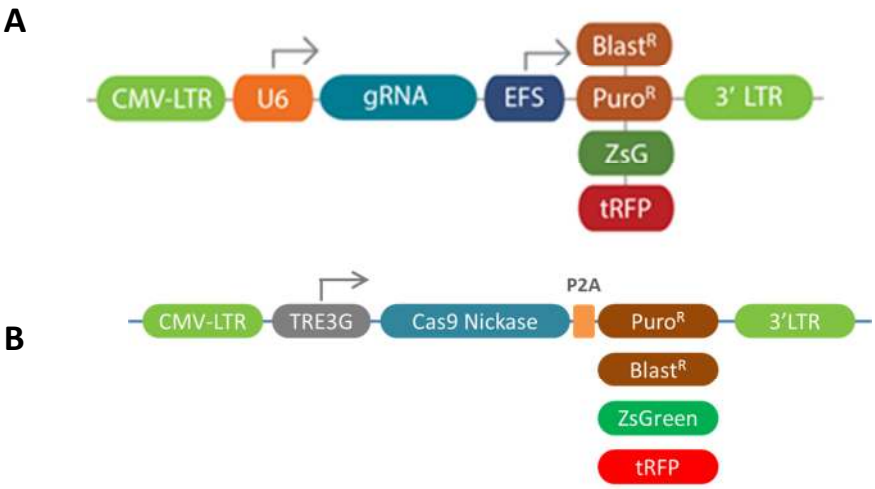

Figure 2. (A) Cartoon of the basic elements of the pCLIP-gRNA lentiviral vectors. Multiple selection markers are available. Vectors shown are pCLIP-gRNA (EFS-Blast), pCLIP-GRNA (EFS-Puro), pCLIP-gRNA (EFS-ZsGreen) and pCLIP-gRNA (EFS-tRFP). (B) Cartoon of Cas9 expression vector option for use with pCLIP-gRNA.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

Transduction Optimization and Functional Titer

This manual provides the protocols for determining the optimal puromycin selection, optimizing transduction and determining relative transduction efficiency. Please review **Error! Reference source not found.** for all safety considerations prior to beginning these protocols.

3. 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. 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 3.

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. Replace the viral supernatant with complete media including Dox and allow induction and growth for 48 hours.
        ii. 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

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Alternate Titering Protocol for Cas9 Vectors

Inducible Cas9 requires a cell line expressing the TET3G transactivator for titering. The expression of the fluorescent and selection markers requires induction of Cas9 for expression. The protocol for creating this cell line and determining the optimal Dox concentration is outlined in detail in Section III.

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. Replace media with media containing Dox to induce marker expression.
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. Replace the viral supernatant with complete media including Dox and allow induction and growth for 48 hours.
        ii. 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 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

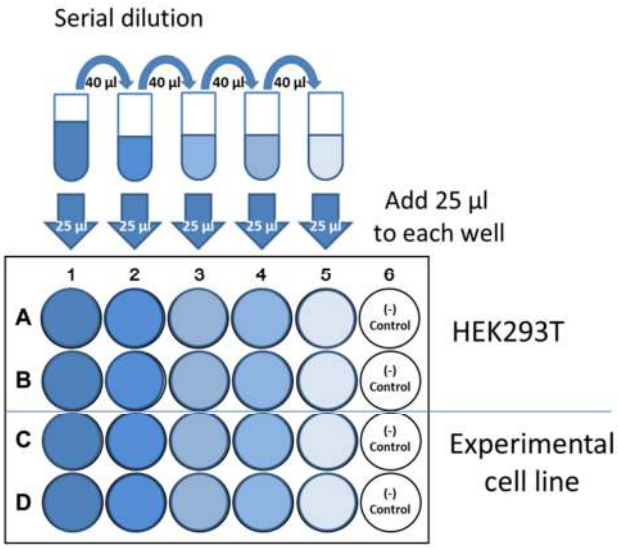


Figure 3 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

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 x 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)

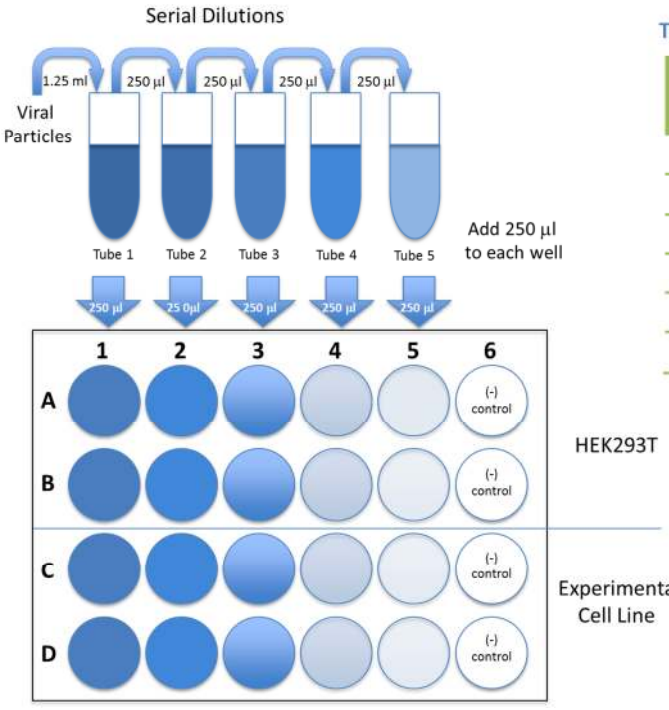


Figure 4 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

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 x 10^4 , column 3 of the plate (1/25 dilution) would be expected to have 100 colonies.

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 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.

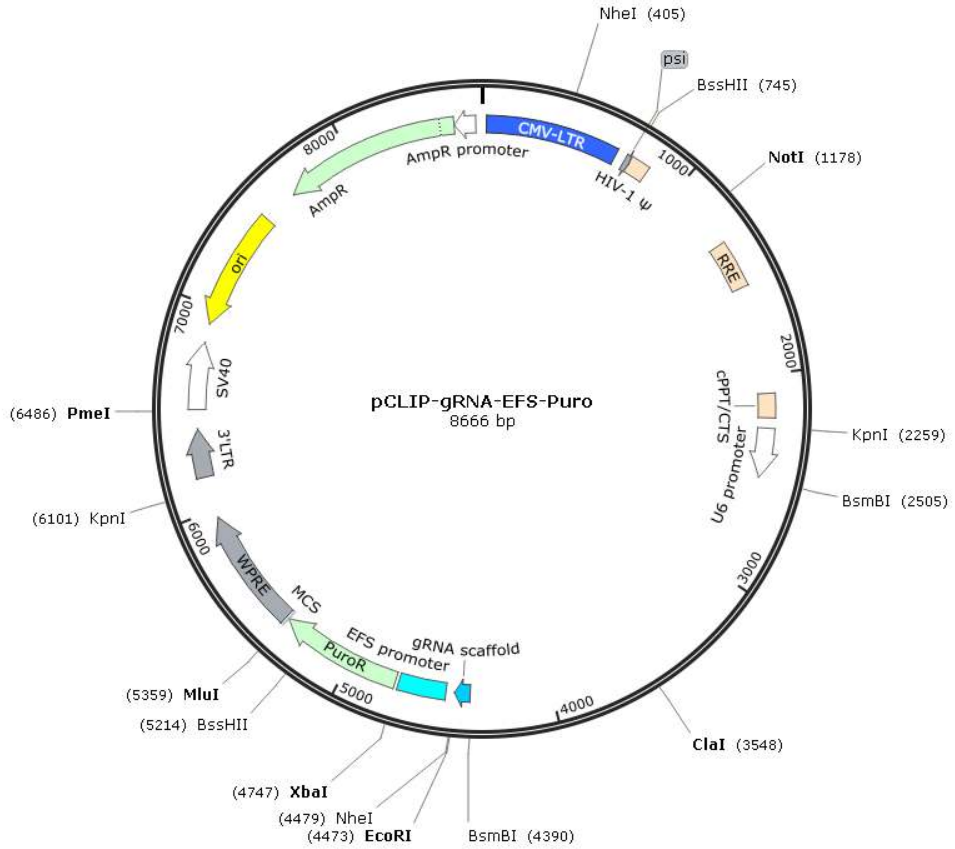
Fluorescence analysis: If the vector expresses a fluorescent protein, incubate for 24-72 hours following transduction and then examine the cells microscopically for fluorescence expression. Sort the cells based on level of fluorescence and select the highest expressing population.

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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