transEDIT-dual™ Lentiviral gRNA CRISPR Pooled Screening Libraries

Vector: pCLIP-dual vectors

Format: Plasmid DNA or lentiviral particles

This manual provides information for the transfection, transduction, and viral packaging of the **transEDIT-dual™** lentiviral gRNA expression vectors (pCLIP-dual). Appendix 1 contains information regarding how to locate the specific vector map for your constructs and Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

Please also refer to the resource publication below.
Erard, N., Knott S., & Hannon, G. Molecular Cell, 2017. "An arrayed CRISPR library for individual, combinatorial or multiplexed gene knockout"

Section 1: Introduction

The **transEDIT-*dual***™ CRISPR arrayed library was developed using the CRoatan algorithm (Erard, Knott and Hannon) by employing a random-forest-based gRNA prediction tool to create novel and superior gRNA designs. These gRNAs were then paired in a lentiviral vector to co-express and thus further augment their potency. The dual gRNAs are provided in a single vector to be used with Cas9 nuclease expression vectors, or cells that already express Cas9, for superior knockout efficiency with increased specificity and sensitivity. A molecular barcode was incorporated in the vector to allow for downstream screening of high throughput analysis. **TransEDIT-*dual***™ Lentiviral CRISPR pooled screening libraries enable high-throughput, multiplexed CRISPR/Cas9 mediated gene knockout/in. The pools can be used to screen hundreds of genes using a standard tissue culture hood without the need for costly automation and liquid handling required for traditional arrayed screens. Lentiviral vectors allow facilitate use in the broadest range of cell lines for functional screens including primary, stem, and non-dividing cells.

Pooled gRNA/CAs9 libraries are typically used to perform enrichment (positive) or dropout (negative) selection screens. Both strategies begin by transducing viral particles expressing a complex pool of gRNAs such that each transduced cell expresses a single gRNA construct. Next, a selective pressure is applied to the heterogeneous population of cells to elicit a desired phenotypic change (e.g. survival screen as shown in **Figure 1**). The gRNA is integrated into the cellular genome and its representation in the culture increases or decreases as the cells are selected for or against, respectively. Changes in the gRNA representation can be determined through sequence analysis of the gRNA sequence.
NOTE: Co-transfection/transduction of a Cas9 vector or use of a stable expressing Cas9 cell line is necessary.

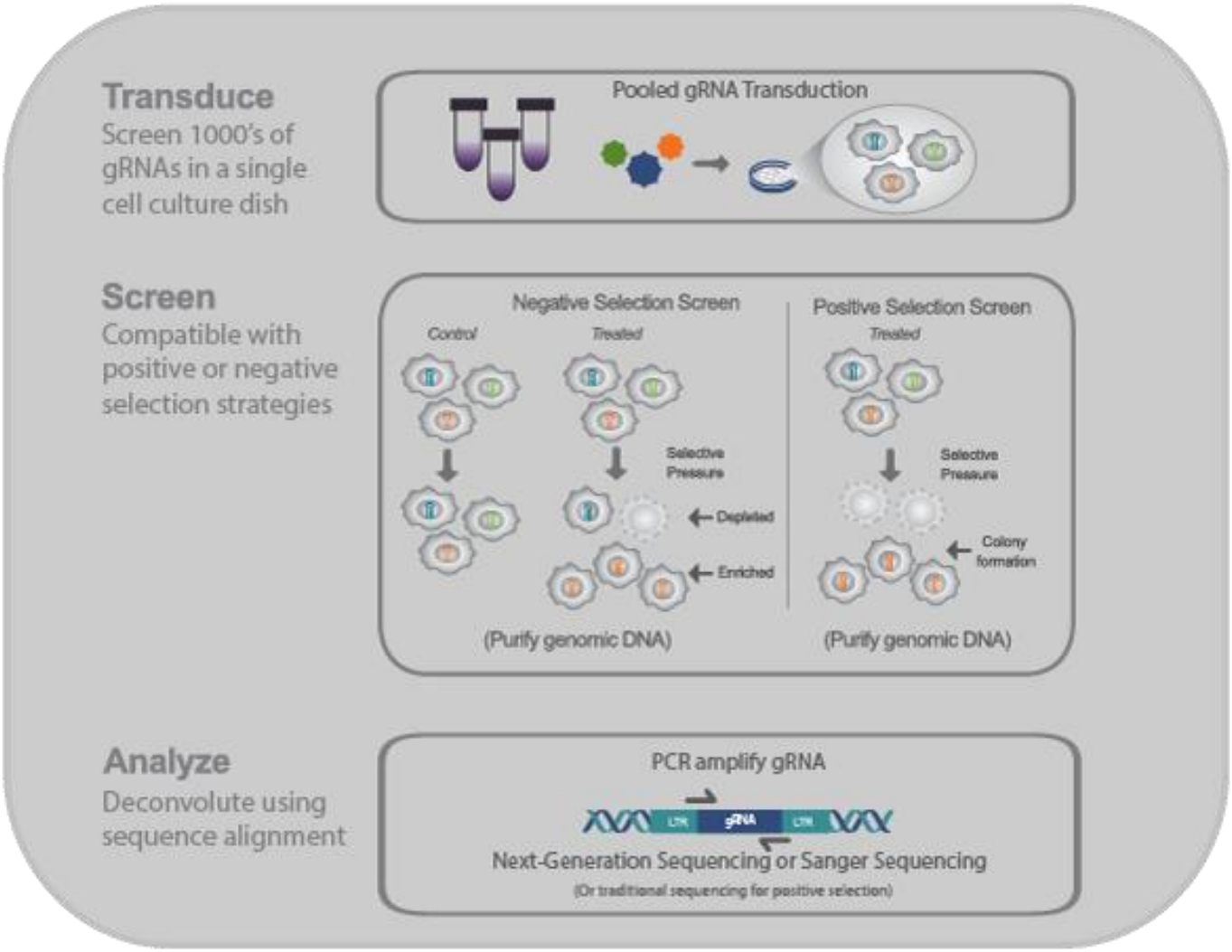


Figure 1. Schematic showing the three basic phases of the screening process: transduction, screening and analysis.

Section 2: Vector information

The pCLIP-dual vectors allow transient and stable transfection; as well as the stable delivery of two gRNA into host cells via a replication-incompetent lentivirus. The pCLIP-dual vector (**Figure 2**) is designed to be used in conjunction with a Cas9 expression vector (**Figure 3**) or cells that already express Cas9.

Vector Highlights:

- Transduction of primary and non-dividing cells
- Fluorescent marker for direct detection (ZsGreen or RFP)
- Selection marker (puromycin or blasticidin)
- Markers allow enrichment of transduced cells and increased genome editing efficiency.

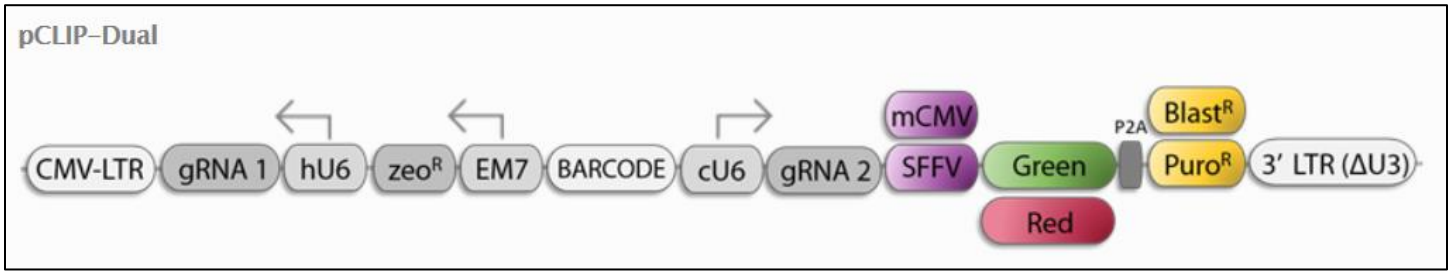


Figure 2. Vector map of the pCLIP-dual gRNA expression vector with the relevant features highlighted. The 2 gRNAs are expressed from a single lentiviral vector that harbors two divergent U6 promoters, a 25bp identification barcode, Illumina®-adapters, choice of mammalian cell promoter, fluorophore, and selection marker. This results in a bicistronic fluorophore-P2A-selection transcript.

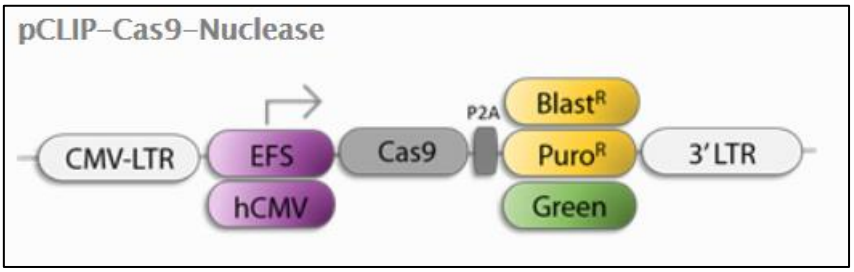


Figure 3. Vector map Cas9 expression vector option for use with pCLIP-dual.

Section 3: Product specifications

Shipping and storage

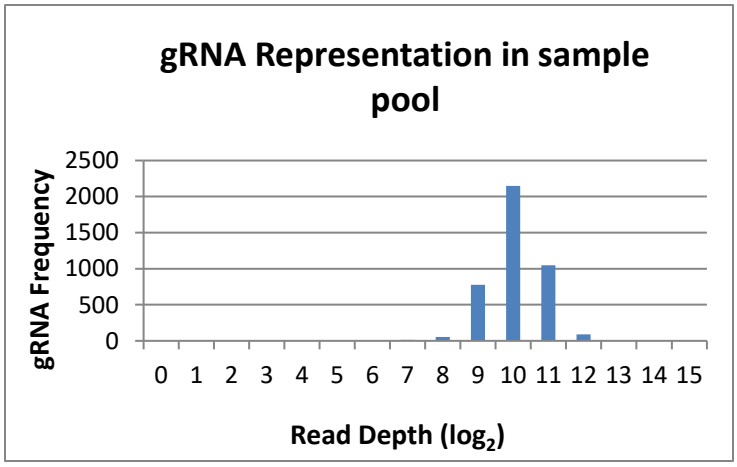
Plasmid components are shipped on wet ice and should be stored at -20°C/-80°C upon receipt.
Viral particles are shipped on dry ice and should be stored at -80°C upon receipt.

Pooled library components

- Pooled Screening Library-Plasmid DNA or Viral Particle format
- Non-targeting control is present in the pool
- Certificate of Analyses are emailed at the time of shipment and can also be requested at info@transomic.com
- **NOTE: pCLIP-Cas9 is sold separately**

gRNA Representation in Pooled Libraries-Quality Control

TransEDIT pools have been developed using strategies that maintain equimolarity of the pooled gRNA constructs – to ensure that screening results are not artificially biased to any one or some gene/s or construct/s prior to the screen. All gRNA/Cas9 plasmid DNA pools are evaluated by next-generation sequencing (NGS) for distribution and representation analysis. Quality metrics are outlined in the Certificate of Analysis provided with each pool. A representative graph of gRNA distribution is shown in **Figure 4.** As the plasmid DNA pools are equimolar, the virus produced from these have the same equimolar representation of gRNAs.



Pool Screening Metrics	
Total gRNA in Pool	5080
gRNA Detected (by NGS)	5004
% Recovery	98.5%
# ± 5X Mean Read Depth	4737
% ± 5X Mean Read Depth	94.7%

Figure 4. Example histogram showing distribution of gRNA from a plasmid DNA pool as seen by NGS analysis (left). Summary statistics of QC analysis (right). The summary includes the total gRNA constructs pooled to create the library and the number detected by NGS.

Section 4: Pooled gRNA Screen-Preparation and Workflow

There are many methods available for CRISPR screening and strategies are constantly evolving. Below is an example workflow.

This guide provides suggested protocols for each step performed during pooled gRNA screening. Please review Appendix 2 for all safety considerations prior to beginning these protocols.

- Antibiotic selection kill curve prior to the pooled screening
- Viral packaging of pool (applicable to plasmid format and Cas9 vector)
- Transfection/Transduction of Cas9 vector into experimental cell line
- Functional titer and transduction optimization in experimental cell line
- Determination of appropriate MOI
- Transduction of pool (and Cas9)
- Enrichment for increased genome editing efficiency
- Primary selection screen
- gRNA amplification and Nextgen sequencing
- Data analysis

Section 5: Selection kill curve

The **pCLIP-dual vector** and **pCLIP-Cas9 Nuclease** vectors have a puromycin or blasticidin resistance marker for selection in mammalian cells (if a fluorophore was not chosen for pCLIP-Cas9-nuclease). Once transfection/transduction has occurred, the cells can be treated to select for cells expressing antibiotic resistance. 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. The same kill curve can be used for both as shown in the example in **Table 1**.

Materials

- Complete media for experimental cell line
- Appropriate antibiotic for selection
    - Blasticidin S HCl antibiotic (Life Technologies, Catalog# A11139-03) (1.25 µg/µl stock solution)
    - Puromycin Dihydrochloride (Life Technologies, Catalog# A11138-03) (1.25 µg/µl 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 antibiotics.
2. Prepare antibiotic dilutions in culture media for titration as shown in Table 1 below:

a Prepare puromycin or blasticidin dilutions in culture media for titration as shown in the example in **Table 1**.

Table 1. Example dilutions and volumes required for establishing optimal antibiotic concentration for puromycin and blasticidin

Volume of Puromycin or 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 antibiotic.
4. Incubate cells with 5% CO_2 at 37°C, or use conditions normal for the target cells.
5. Check cells daily to estimate rate of cell death.
6. Replenish the media containing the appropriate concentrations of antibiotic every 2 days for 6 days.

Note: The optimal antibiotic 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.

Section 6: Transfection of transEDIT CRISPR/Cas9 Vectors

The experimental cells must express Cas9 before the pool is added. This can be done by either transfecting with the Cas9 vector, or transducing with Cas9 virus. (Refer to **section 7** for viral production).

Use the following procedure to transfect Cas9 vector 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**)

The CRISPR pool should be packaged into viral particles (Refer to **section 7** for viral production). TransEDIT CRISPR pools are also available for purchase in viral particle format.

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)
- Puromycin Dihydrochloride (Life Technologies, Catalog# A11138-03)
- Sterile 1.5 ml microfuge tubes
- Assays for assessing genome editing (e.g. Surveyor Assay, sequencing etc.)

Equipment

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

Transfection complex preparation for pCLIP-Cas9-nuclease (Figure 5)

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 48-96 hours changing media as necessary.
3. Perform assay to determine genome editing efficiency.

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

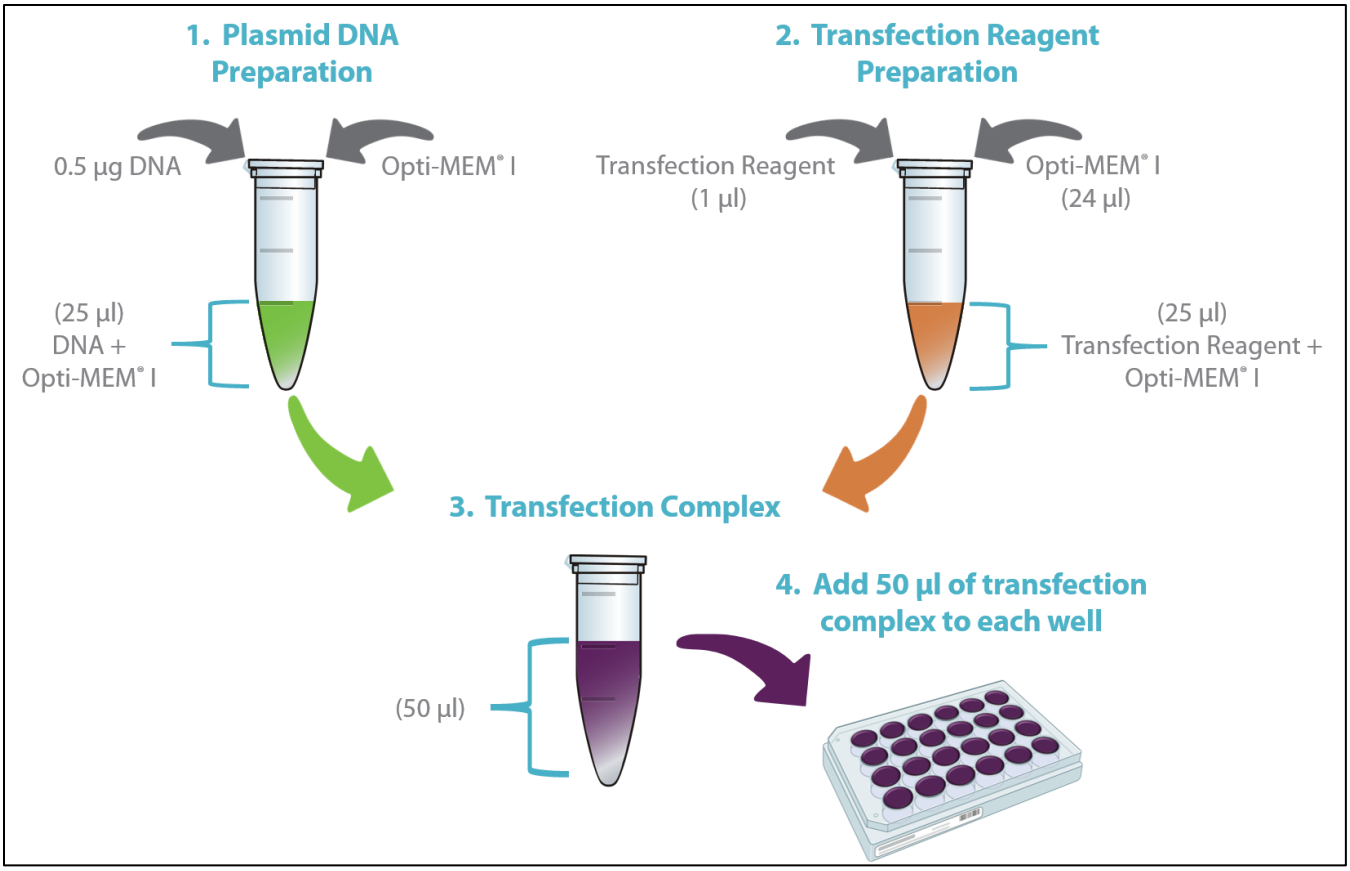


Figure 5: Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24.

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 the transfection.
4. Assay cells for gene activity 24 hours following the addition of transfection complex to cells.

Selection after transfection- Enrichment for increased genome editing efficiency

After 24-48 hours, Cas9 transfected cells can be selected using antibiotic resistance or fluorescent protein expression. The **transEDIT™ pCLIP-Cas9-nuclease** lentiviral vectors express either a fluorophore (ZsGreen) or a mammalian selection marker (puromycin or blasticidin), depending on the vector chosen. 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.

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 with 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: To assay for 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. 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 fluorescent protein, 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.

Section 7: Packaging Lentiviral Particles

Some cell lines are resistant to transfection. Lentiviral particles offer an alternative delivery method. The **transEDIT™ pCLIP-dual** and **pCLIP-gRNA vectors** can be packaged into lentiviral particles for efficient delivery into target cell lines. Constitutive vectors may be packaged with 2nd, 3rd, or 4th generation packaging plasmids.

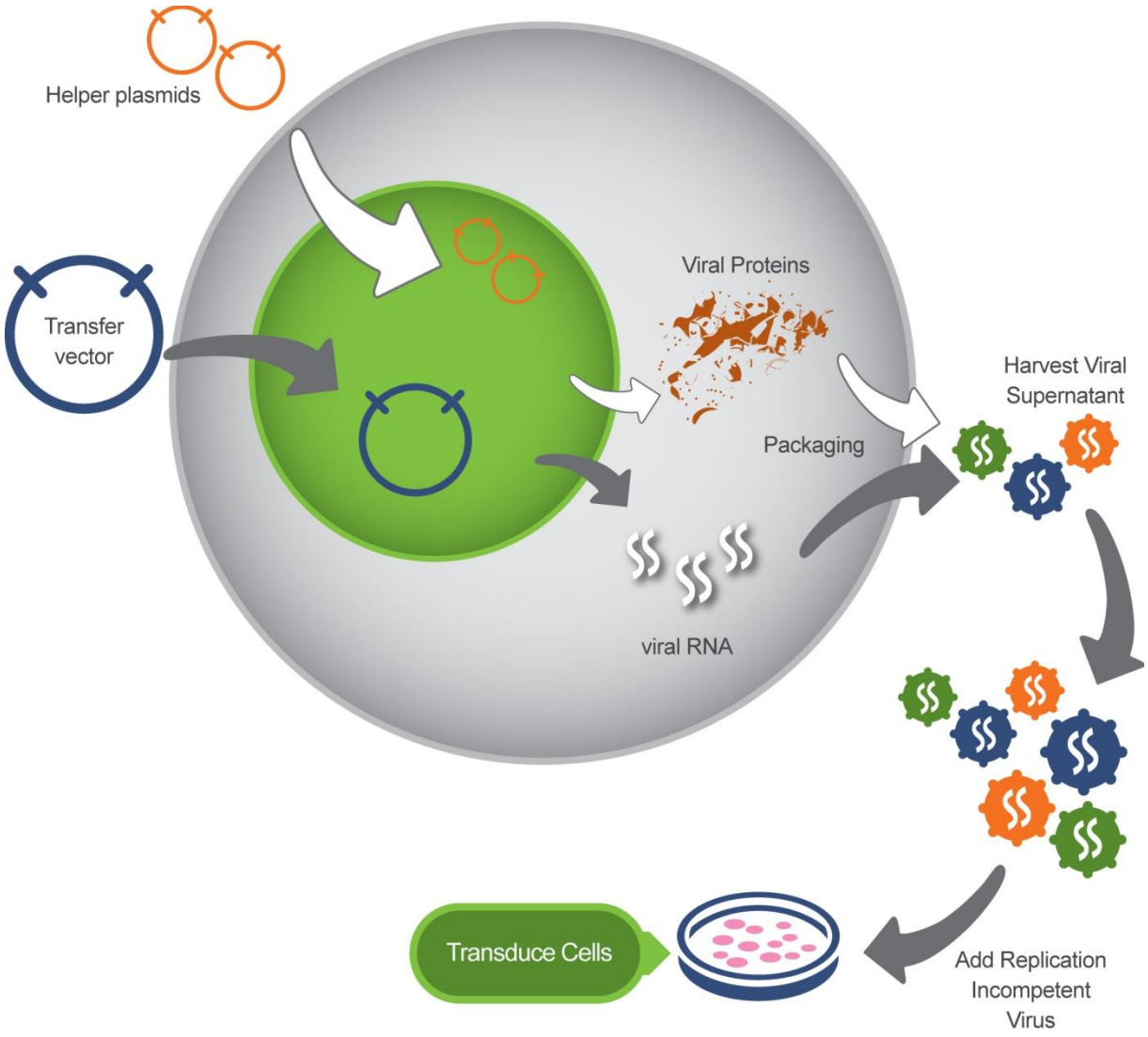


Figure 6: Schematic depicting lentiviral packaging of lentiviral vectors

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 (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 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-dR8.2 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)
- 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 for packaging pCLIP-dual and Cas9 vectors

1. Plate the target cells and HEK293T cells 18-24 hours prior to transfection in a 6-well tissue culture 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 CRISPR/Cas9 plasmids and lentiviral vector packaging mix for transfection (Note: all plasmids are re-suspended in dH_2O):
    a. Transfer vector (pCLIP-Cas9 Nuclease or pCLIP-dual) – dilute plasmid to 0.2 μg/μl
    b. Lentiviral packaging mix (0.5 μg/μl):
        i. 100 μl pCMV-dR8.2 (0.5 μg/μl)
        ii. 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:
    a. Add 5 μl of transfer vector (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 ℃ 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 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 the 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.

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 (harvested or purchased)
- 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 the 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 the target cells and HEK293T cells 18-24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 12 wells for each cell line with complete media (see **Figure 7**). 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 7**.
   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.
   b. Fluorescent colony counting
      i. Replace the viral supernatant with complete media and allow growth for 48 hours.
      ii. Count the number of colonies expressing the fluorophore. A colony consisting of multiple cells should be counted as a single transduction event.

10. Use the calculation below and **Table 3** to determine functional titer. (Alternate methods for calculating are described in Appendix 3.)

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

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

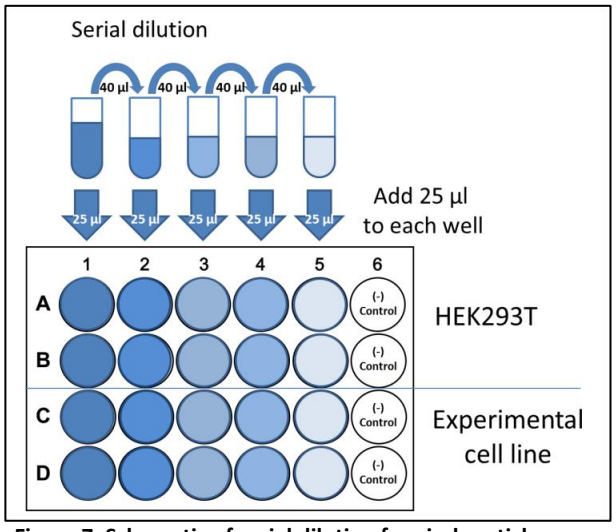


Figure 7. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

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 fluorescent colonies for a viral titer of 5 x 10^5 TU/ml 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	100,000	20,000	4,000	800	160	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent 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:

$$TU_{total} = (MOI \times Cell\ Number)/Viral\ titer\ (TU/\mu l)$$

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
- Fluorescent microscope with appropriate filter

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. Using a fluorescent microscope, assess fluorescent expression in the wells.

> **Note**: Levels of fluorescent protein expression will vary greatly across a culture due to random integration of lentiviral vectors into regions of the chromosomes with varying levels of transcriptionally active and non-active states.

Section 10: Selection after transduction- Enrichment for increased genome editing efficiency

After 24-48 hours, transduced cells can be selected using antibiotic resistance or fluorescent protein expression. **pCLIP-dual vectors** have puromycin or blasticidin selection and a ZsGreen or RFP fluorophore while the **transEDIT™ pCLIP-Cas9-nuclease** lentiviral vectors express either a fluorophore (ZsGreen) or a mammalian selection marker (puromycin or blasticidin), depending on the vector chosen. 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 antibiotic 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 with the appropriate antibiotic.
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: To assay for 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 fluorescent protein, 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.

Section 11: Primary Selection Screen: Recommendations & Considerations

Once functional titer, transduction efficiency, and optimal antibiotic concentration have been evaluated in the experimental cell line, the primary selection screen can be performed. The following protocol provides recommendations and guidelines to perform the selection screen.

Assay optimization

The objective of a pooled screen is to selectively enrich or deplete cells in a population based on phenotypic/molecular change. Biological assays used to evaluate phenotypic/molecular changes in the pooled screen may take different forms including survival, behavior (e.g. migration or adhesion), changes in reporter expression or changes in surface marker expression. Each will have variables inherent to the assay that should be optimized such as sensitivity of the assay, concentration of selective agent, duration of treatment, and method of selection.

For Example:
Selection screens identify genes affecting cell viability. If a targeted gene decreases growth by 10%, these cells will show a 40% depletion in the population on day 5, but an 80% depletion on day 15. So, it is possible to detect more subtle changes simply by extending time points.

It is also critical to understand gRNA fold representation within the assay. The average number of cells independently transduced with constructs expressing the same gRNA is described as gRNA fold representation. Transductions with high fold representation (e.g. 500X – 1000X) and uniform representation for every gRNA provide the optimal starting point for a screen. Higher representation maintains the uniformity across the pool and increases reproducibility between biological replicates. At least two biological replicates are recommended per screen.

Multiplicity of Infection (MOI)

In a pooled screen it is critical that transduction is optimized to ensure that each transduced cell has a single genomic integration from the gRNA-expressing viral particle. This enables one to clearly see phenotypes associated with the knockdown of the single gene. The number of integrations correlates with multiplicity of infection (MOI) calculated as the ratio of transducing units to cells. At an MOI of 0.3, the majority of infected cells are predicted to have a single integration and is therefore recommended for pooled screening. The average number of integrations is estimated by the Poisson distribution (**Figure 8**). The number of cells needed at transduction is determined using the number of cells with viral integrants and the desired MOI (see calculation below).

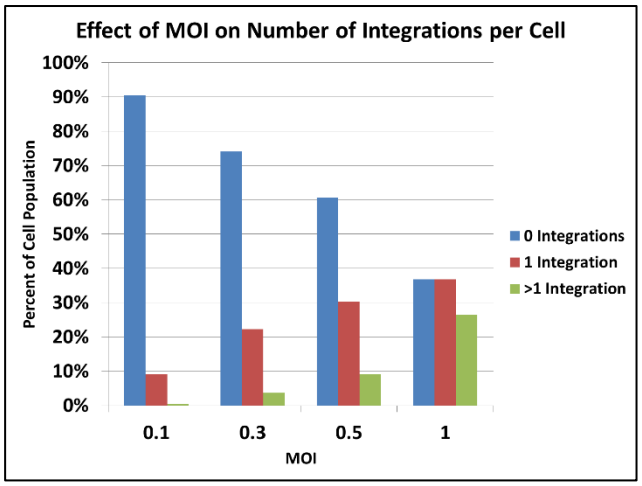


Figure 8: Higher MOIs result in a higher number of cells with multiple integrations. An MOI of 0.3 is recommended for pooled screening to limit the number of cells with multiple integrations (4%) and limit the number of cells required for transduction.

Calculations for MOI

Number of viral integrants needed:

Number of gRNA in the pool x Fold representation = Number of integrants needed

Number of cells needed at transduction:

Number of integrants needed ÷ MOI = Number of cells needed at transduction

For Example:
In a pooled screen, cells should be transduced at an MOI of 0.3 to maximize the number of cells with a single integration and limit the number of cells needed at transduction. Transducing a pool of 500 gRNA at 1000-fold representation will require $5x10^5$ transduction units (TU) and approximately 1.5×10^6 cells to achieve an MOI of 0.3.

Calculate as follows:

500 gRNA x 1000 fold representation = 5×10^5 TU

5×10^5 TU / 0.3 MOI = 1.6×10^6

Viral particles in transduction

Prior to your screen, confirm that there is sufficient volume of viral particles for the biological replicates and representation needed for your experimental design. The total number of TU needed for the experiment and titer will be required to calculate the volume needed for the experiment. Refer to the titer calculated in previous sections.

Number of transducing units:

Representation x gRNA per pool x Biological replicates = Total TU needed for experiment

Total volume of virus needed for experiment:

TU for experiment ÷ Functional Titer in Experimental Cell Line (TU/ml) = Volume (ml) of virus needed for the experiment

Section 12: Primary Selection Screen: Guidelines & Protocols

The primary selection screen protocol provides a basic outline of the transduction process. The following should be optimized before proceeding to the primary selection screen:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml appropriate antibiotic for selection
- Number of live cells that should be plated when splitting
- Number of cells that should be harvested for each sample

Material

- Complete media for experimental cell line
- Complete media for experimental cell line with appropriate antibiotic for selection
- Pooled screening library as viral particles
- Assay specific controls and reagents
- Qubit® dsDNA BR Assay or Quant-iT™ PicoGreen ® dsDNA Kits (or other fluorometric system specific for quantitation of dsDNA)

Equipment

- Automatic pipette /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment
- Qubit® fluorometer or standard spectrofluorometer with fluorescein excitation and emission wavelengths

Protocol

Prepare cells

1. Plate cells such that they are actively dividing and 40 - 60% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.

Transduce cells

3. Exchange media with transduction media.
   Note: Media should be serum free for maximum transduction efficiency.
4. Incubate cells 12 - 24 hours in transduction media.
5. Replace transduction media with complete media (no antibiotic selection).

Antibiotic selection

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

Sampling and passaging of cells during screen

9. Split the culture into a control sample and an experimental sample. Each sample should be derived from enough cells to maintain representation of the pool (500-1000 times the number of gRNA in the pool).

10. Start assay selection and take samples as needed. Each sample should include enough cells to maintain representation of the pool (500-1000 times the number of gRNA in the pool).

Genomic DNA extraction

Extract genomic DNA from samples. Ensure that genomic DNA is extracted from enough cells to maintain representation of the pool (500-1000 times the number of gRNA in the pool). The publication recommends QIAGEN QIAamp DNA Blood Midi kit.

Please refer to the publication:

Erard, N., Knott S., & Hannon, G. Molecular Cell, 2017. "An arrayed CRISPR library for individual, combinatorial or multiplexed gene knockout"

> **Note:** Subsequent primary PCR should be performed on all extracted genomic DNA. To ensure that representation is not lost during the genomic DNA purification step the maximum column capacity should not be exceeded as this can decrease yields. In addition, a second elution from the column preparation may be necessary to maximize yields.

11. Quantitate genomic DNA isolated from each sample and ensure that 260/280 ratios are greater than or equal to 1.8.

For example:
A pool containing 1500 gRNA used for screening with coverage of 1000 viral integrants per gRNA would require a total of 1.5×10^6 transduced cells. To maintain the 1000-fold coverage of gRNA used during the screen, at least 1.5×10^6 cells are needed for genomic DNA isolation from each sample.

Section 13: gRNA Amplification and Next Generation Sequencing (NGS)

Note: If doing a positive selection screen, regular Sanger sequencing can be performed instead of NGS.

The representation of each gRNA is detected by next generation sequencing (NGS). The gRNA sequence integrated into the targeted cells genome can be amplified using common sequences flanking the gRNA and Illumina® indexes are added to each sample. The pooled PCR product from all samples may be analyzed in parallel using NGS analysis. Index primers can be ordered from Eurofins or Integrated DNA Technologies. See **Appendix 4** for primers.

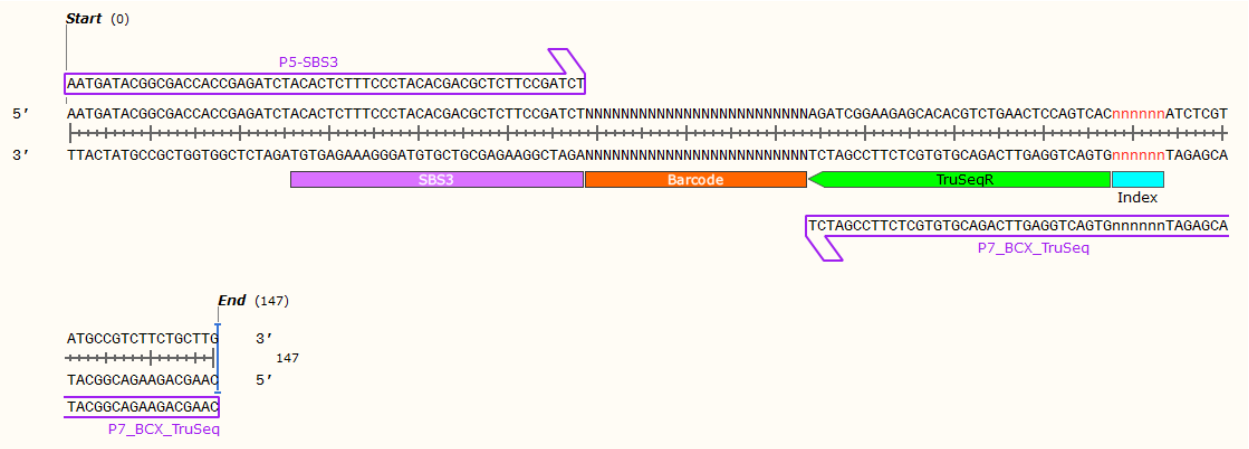


Figure 9. Individual barcodes are amplified from experimental genomic DNA with a single round of PCR. The PCR amplifies the barcode unique to the individual pCLIP-dual construct and adds adapters for use on Illumina® sequencing platforms.

PCR guidelines

PCR reactions should be carried out with no more than ~850 ng of genomic DNA per 50 µl reaction; therefore, multiple PCR reactions will be required per sample. Calculate the total ug amount of genomic DNA needed for PCR. If the calculated ug amount is not reached, PCR all extracted genomic DNA to maintain representation of gRNA coverage used during the screen of the pool (500-1000 times the number of gRNA in the pool). See the example below for calculations.

For example:
A pool containing 1500 gRNA used for screening with coverage of 1000 viral integrants per gRNA would require 9.9 µg of genomic DNA (genomic DNA) per sample be used for amplification.

- 1500(gRNA) x 1000(coverage) x 6.6^{-12} (g/diploid genome)= 9.9^{-6} grams genomic DNA (or 9.9µg)
  Each 50 µl PCR reaction will contain 850 ng of genomic DNA.
- 9.9 µg (or 9900 ng) / 850 ng DNA = 11.6 reactions
- Twelve PCR reactions per sample will be required for genomic DNA amplification from this pool.

Materials

- KOD Hot Start Polymerase (Millipore Sigma Cat# 71086-4)
- 96 well PCR plates
- Agarose gel
- Qubit® dsDNA BR Assay or Quant-iT™ PicoGreen ® dsDNA Kits (recommended for their specificity to quantitate dsDNA in solution)
- BECKMAN COULTER Ampure XP beads

Equipment

- Qubit® fluorometer or standard spectrofluorometer with fluorescein excitation and emission wavelengths
- NGS platform

PCR Protocol

***Note**:* KOD Hot Start Polymerase, Millipore Sigma Cat# 71086-4 is recommended for PCR. See **Appendix 4** for PCR primer sequences. Samples to be analyzed on the same run should use their own uniquely indexed reverse primer.

Components	µl per reaction
10X KOD Buffer	5
dNTP Mix (2 mM each)	5
$MgSO_4$ (25 mM)	4
Betaine (5 M)	5
Primary PCR Forward (10 µM)	1.7
Primary PCR Reverse (10 µM)	1.7
KOD Hot Start Polymerase	1.5
Genomic DNA (850 ng)	--
Nuclease free water	--
Total Volume	50

98C	5min
25cycles of	
95C	30sec
60C	30sec
72C	30sec
72C	5min
4C	hold

1. Based on the genomic DNA quantitation determine the total number of reactions required to amplify genomic DNA using no more than 850 ng of genomic DNA per 50 µl PCR reaction.

2. Set up PCR reactions using components and volumes outlined above for each sample. A 96-well PCR plate is recommended.
3. Place samples in the thermal cycler with heated lid on and run the PCR program.
4. After cycling is complete, briefly centrifuge the 96 well plate containing PCR reactions.
5. Select at least 4 PCR reactions per sample and run 10 µl from each on an agarose gel to verify the **147bp** amplicon.
6. Pool PCR reactions from each sample and purify following manufacturer's protocol. *BECKMAN COULTER Ampure XP beads kit is recommended.*

Preparing NGS adapted Amplicons for sequencing

After secondary PCR and purification, it is necessary to accurately quantitate each sample prior to NGS analysis. Quantitate purified PCR reactions using a fluorometric assay specific for double stranded DNA (dsDNA). Consult your sequencing facility for dilution and denaturing of libraries for NGS on an Illumina platform. It is recommended that you achieve 500-1000X reads relative to the number of constructs in your pool.

Data Analysis

Analysis of NGS data from a gRNA screen is complex and can be difficult to evaluate without the proper experience or guidance. There are many web-based tools that can be used for alignment and analysis of NGS data; however, for optimal analysis with viable hit identification, it is recommended to have screen data analyzed by an expert in the field of NGS and bioinformatics. Once data has been analyzed and gRNA representation has been identified as statistically significant between control and experimental samples, secondary validation of these hits by gRNA should be performed for confirmation of hits. Individual gRNA constructs can be used for hit validation. In many cases, further validation can be performed from over expression as well.

Appendices

Appendix 1 – Vector maps

Full vector sequences and maps are 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 - Methods for titering

1. Antibiotic 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 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 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. Fluorescent 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.

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

Appendix 4 - PCR and NGS sequencing primer sequences

Note. Highlighted Index sequences are the reverse compliment to the Illumina low-throughput Indices.

Forward Primer	P5-SBS3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
Reverse Primer	P7_BC1_TruSeq	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC2_TruSeq	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC3_TruSeq	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC4_TruSeq	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC5_TruSeq	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC6_TruSeq	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC7_TruSeq	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC8_TruSeq	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC9_TruSeq	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Reverse Primer	P7_BC10_TruSeq	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC11_TruSeq	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Reverse Primer	P7_BC12_TruSeq	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Appendix 5 – References and recommended reading

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