transEDIT™ pCLIP-All-EFS-Puro (Lentiviral gRNA/Cas9) Epigenetics CRISPR Screening Library

Plasmid DNA or Lentivirus Format
CAHD9001, CAHV9001

This manual provides information for the transfection, transduction and viral packaging of the transEDIT lentiviral gRNA expression vector (pCLIP-All-EFS-Puro). 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) and 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 (gRNAs), 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.

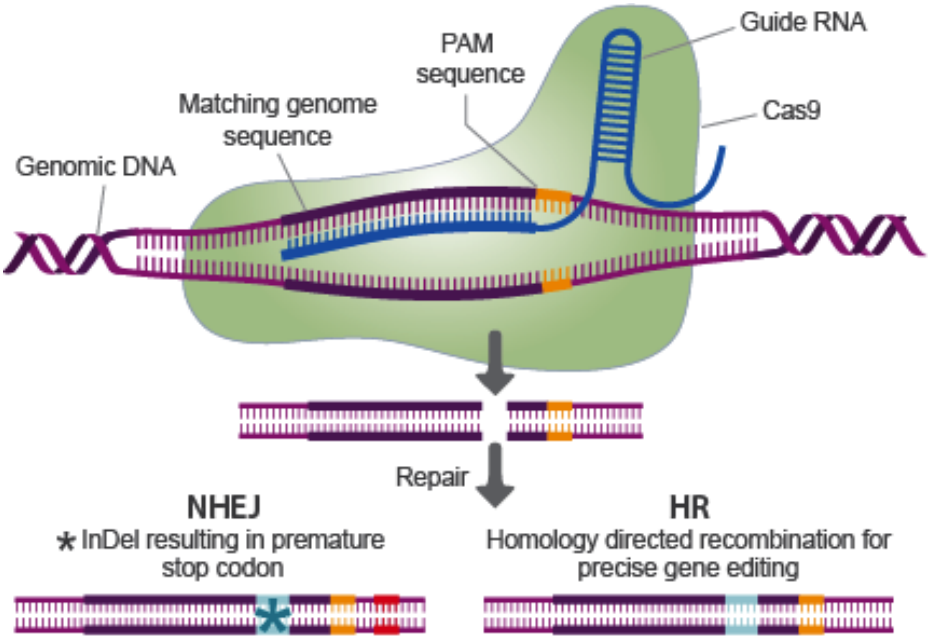


Figure 1. Schematic representation of RNA-guided double-stranded DNA cleavage by CRISPR/Cas9 using a programmable guide RNA.

2. Screen with CRISPR/CAS9 Pools

The pCLIP-All Epigenetics CRISPR Screening Library from transOMIC technologies are pooled lentiviral constructs expressing gRNAs targeting human epigenetic genes to enable highly multiplexed, CRISPR/Cas9 mediated gene knockout/in. Screening libraries are available as pooled plasmid DNA or pooled lentiviral particles.
The pCLIP-All Epigenetics CRISPR Screening Library can be used to screen hundreds of genes in a high-throughput, multiplexed assay using a standard tissue culture hood without the need for costly automation and liquid handling required for traditional arrayed screens. Lentiviral vectors allow the user to select from the broadest range of cell lines for functional screens including: primary, stem and non-dividing cells.

The transEDIT Lentiviral gRNA/Cas9 Epigenetic Screening library is a pool of lentiviral constructs expressing gRNAs and Cas9 targeting coding epigenetic genes for knockout. Gene knockout pooled screening can be performed to identify the function of a gene in the regulation of cellular responses and signaling pathways. The transEDIT lentiviral gRNA/Cas9 pooled screening platform is available either as a dual vector or single vector system. The dual vector system utilizes a single lentiviral vector for Cas9 expression and a gene-specific lentiviral vector for gRNA expression designed to the target sites of interest. The single vector system uses the pCLIP-ALL lentiviral vector that contains both the Cas9 and the gRNA in the same vector. transEDIT lentiviral epigenetic gRNA pooled screening libraries are comprised of ten or more pre-designed gene specific gRNAs per gene, against ~ 500 epigenetic genes (Shalem *et al.* (2014)).
Lentiviral pooled gRNA screening libraries are provided standard in the pCLIP-gRNA-EFS-Puro or pCLIP-All-EFS-Puro vectors (For the pCLIP-gRNA-EFS-Puro vector please refer to the alternative manual). They are also available in the pCLIP-All-EFS-Blast/ZSGreen/tRFP). **No changes to the protocols are required for use with any of the alternate pCLIP-ALL vectors.**
Alternatively, they are available in other pCLIP-gRNA vectors with Blast/ZSGreen/tRFP - please refer to the alternative manual.

Pooled gRNA Screening Strategies

Pooled gRNA/CAs9 libraries are typically used to perform enrichment (positive) or dropout (negative) selection screens. Both strategies by transducing viral particles expressing a complex pool of gRNAs in the pCLIP-ALL such that each transduced cell expresses a single gRNA. 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 2**). The gRNA and Cas9 are 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.

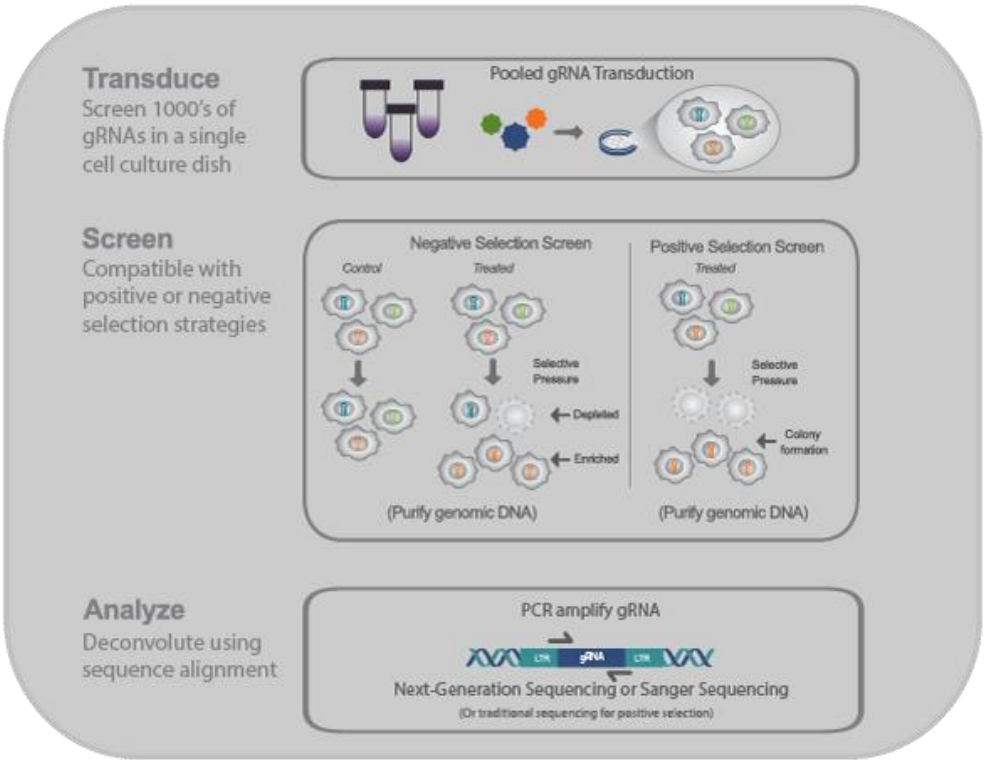


Figure 2. Schematic showing the three basic phases of the screening process: transduction, screening and analysis.

3. Lentiviral pCLIP-All-EFS-Puro vector information

The pCLIP-Clip-All expression vector allows for the stable delivery of gRNA and Cas9 into host cells via a replication-incompetent lentivirus.

- Transduction of primary and non-dividing cells
- Fluorescent red or green markers for direct detection of expression
- Puromycin or blasticidin resistance or fluorescent marker expression for enrichment of transduced cells and increased genome editing efficiency, via antibiotic selection and/or FACS sorting.

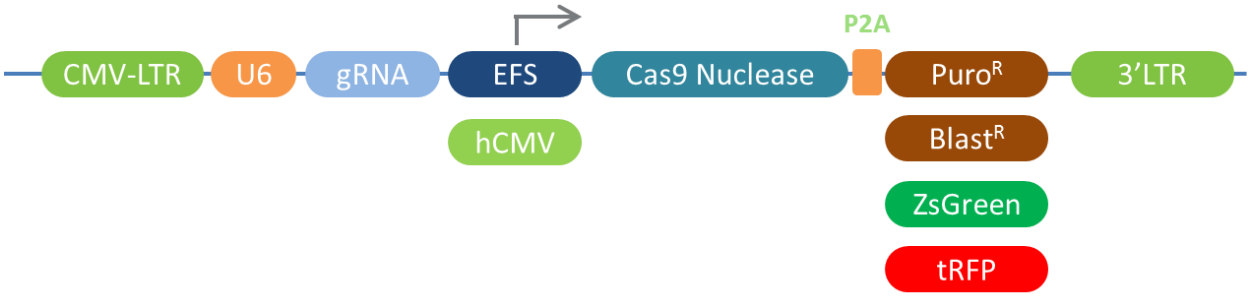


Figure 3 Cartoon of the basic elements of the pCLIP-All lentiviral vectors. Multiple selection markers are available. Vectors shown are pCLIP-All (EFS-Blast), pCLIP-All (EFS-Puro), pCLIP-ALL (EFS-ZsGreen), pCLIP-All (hCMV-tRFP), pCLIP-All (hCMV-Blast), pCLIP-All (hCMV-Puro), pCLIP-ALL (hCMV-ZsGreen) and pCLIP-All (hCMV-tRFP).

4. 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

Plasmid DNA library components

Table 1. Components provided

Component	Quantity	Amount
Pooled Screening library - plasmid DNA	1	50 µg
Non-targeting Control plasmid DNA	1	25 µg
Primary PCR Forward Primer	2	400 µl at 10 µM
Primary PCR Reverse Primer	2	400 µl at 10 µM

Equipment and reagents to be supplied by user

For packaging: *Note: Pools can also be packaged with other systems.*

- OMNIfect™ transfection reagent (transOMIC, Cat. # OTR1003)
- Complete culture medium (DMEM + 10% FBS, 1X Pen/Strep, and 1X L-glutamine)
- OPTI-MEM I + GlutaMAX-I (Gibco, Cat. # 51985-034)
- Transfer vector(s) diluted to 0.2 µg/µl
- Lentiviral packaging mix (0.5 µg/ µl):
  - 100 µl pCMV-dR8.2 (0.5 µg/µl) - (Addgene)
  - 50 µl pCMV-VSV-G (0.5 µg/µl) - (Addgene)
- 10 cm tissue culture plates
- Filter, 0.45 µm made of cellulose acetate, or polysulfonate (low protein binding)
- Automatic pipette/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

For titering:

- 1.5 ml microcentrifuge tubes
- HEK293T cells (NIH-3T3 may be used as an alternative)
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate

For Puromycin kill curve:

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution)
- 24-well tissue culture plate

For Transduction:

- Polybrene
- Puromycin

For PCR:

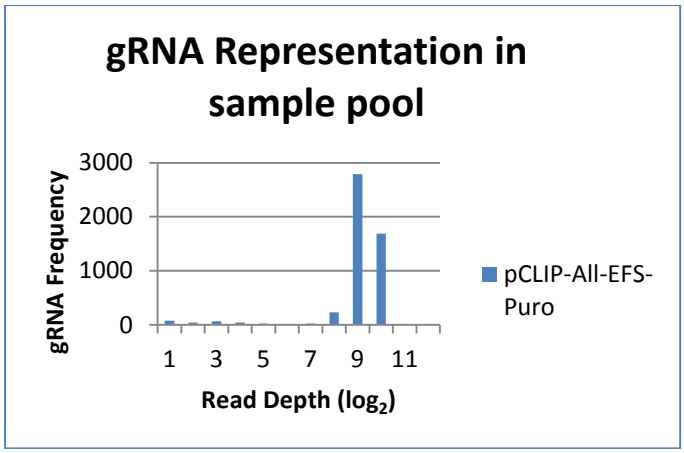
- KOD Hot Start Polymerase (Novagen Cat# 71086-4)
- 96 well PCR plates
- Betaine
- Agarose gel
- PCR purification column (*QIAquick PCR Purification Kit, Qiagen Cat# 28104*)
- DNA gel extraction kit (*QIAquick Gel Extraction, Qiagen Cat# 28104)*
- Multiplex indexed primer kit (12-plex, TRP0001)

Equipment:

- Automatic pipette/Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Fluorescent microscope with GFP filter (refer to Appendix 3 for alternative methods that do not require this)
- Qubit® fluorometric
- Illumina® sequencing platform

5. gRNA representation in pooled libraries – Quality Control

gRNA/Cas9 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 (C of A) provided with each pool. A representative graph of gRNA distribution is shown in Figure 4. For a more detailed vector map and link to vector sequence see Appendix 1. 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 MRD	4737
% ± 5X MRD	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.

6. Pooled gRNA/Cas9 Screen – Preparation and optimization

There are many methods available for CRISPR screening and are constantly evolving.

The following User's 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.

Note: if a fluorescent protein is chosen the kill curve need not be done.

7. Selection kill curve

The optimal antibiotic concentration should be determined for a cell line prior to transduction. The pCLIP-All Nuclease vector have an option for puromycin or blasticidin selection in mammalian cells. These can be used to select cells for expression of plasmids. To establish stable cell lines, once 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-transduction) should be determined. In the following protocol, the lowest concentration that provides adequate selection is determined for the experimental cell line.

Required materials

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution) or
- Blasticidin (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 puromycin.

Prepare puromycin dilutions in culture media for antibiotic titration as shown in **Table 2**.
Use a puromycin stock solution of 1.25 µg/µl stock solution.

Table 2. 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.

8. Transfection

transEDIT CRISPR/Cas9 Vectors

What is in the kit:

- transEDIT Cas9 Vectors (pCLIP-All, glycerol or virus) containing gRNA designed against genes known to have an epigenetic function
- transEDIT pCLIP-All Negative Control (bacterial glycerol stock or virus).

The pCLIP-All pool needs to be made into virus, so will need to be transfected into a packaging cell line.

Use the following procedure to transfect plasmid DNA into mammalian cells in a 24-well format. For other plate formats, scale up or down the amounts of DNA and OMNIfect reagent proportionally to the total transfection volume (**Table 3**). 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.

For Virus production see **section 9**.

Additional Materials

- 24-well tissue culture plates
- Cell culture complete medium for maintenance and passaging of experimental cell line (including serum and supplements)
- OMNIfect™ transfection reagent (transOMIC, Cat# OTR1003)
- 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 (Thermo Fisher, Cat# A11139-03)
- Puromycin Dihydrochloride (Thermo Fisher, Cat# A11138-03)
- Sterile 1.5 ml microfuge tubes
- Assays for assessing genome editing (e.g. Surveyor Assay)

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 (Figure 5):

Volumes and amounts are for each well to be transfected.

1. Plasmid DNA preparation: Dilute 0.5 µg of pCLIP-All Nuclease 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 µl of OMNIfect 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 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.*

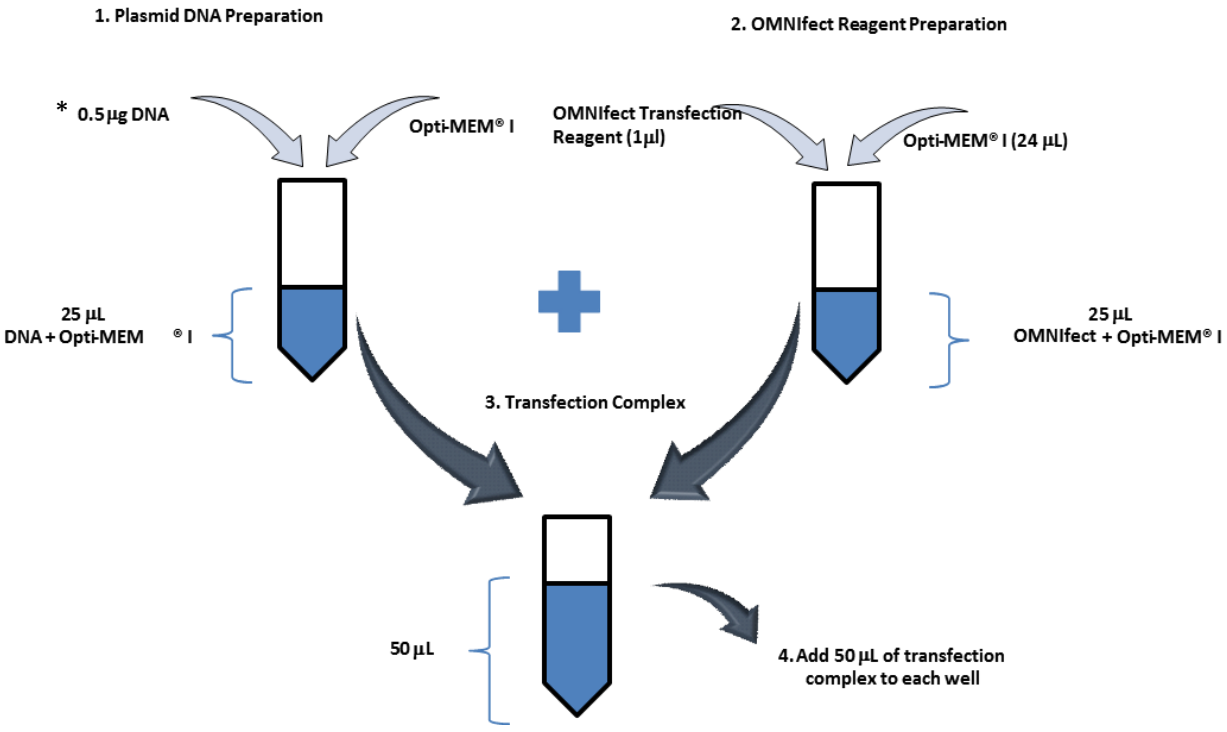


Figure 5. Transfection protocol for 24 well plates (volumes indicated are per well). To transfect the entire plate multiply all volumes and DNA amount by 24. *0.5 µg of DNA in step one represents the 0.5 µg of pCLIP-All plasmid.

Table 3. 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 (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 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.

9. Packaging lentiviral particles for pCLIP-All (if plasmid DNA was purchased)

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

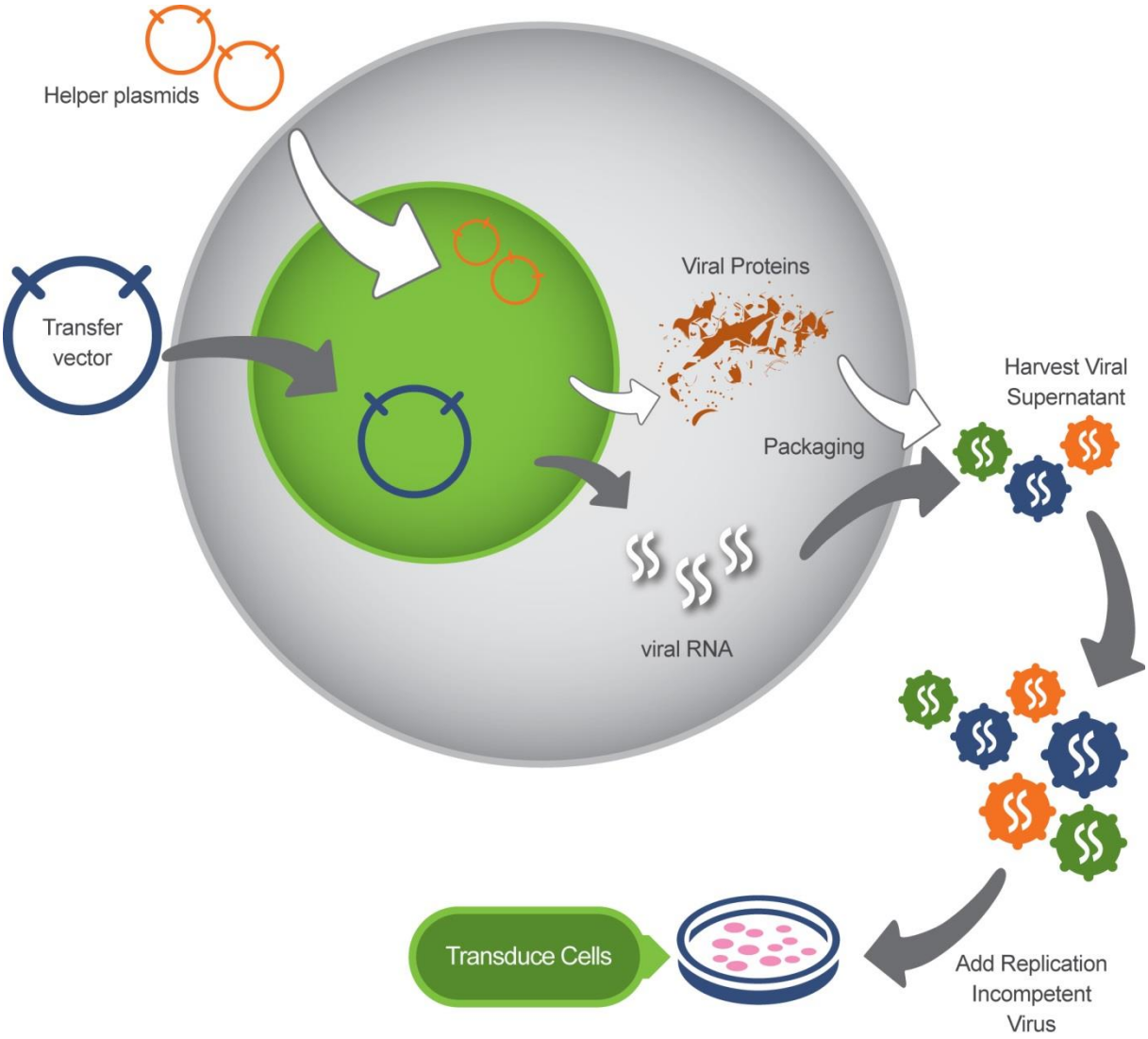


Figure 6. Schematic depicting lentiviral packaging of lentiviral vectors

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

Protocol (pCLIP-All)

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 dH_2O):
    a. Transfer vector (pCLIP-ALL) – 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 pCLIP–All (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: The 50 μl aliquots will be used in the functional titering protocol. They should be stored at -80°C overnight prior to titering to reflect any loss of function due to freeze/thaw cycle that will occur for the transduction aliquots. Freshly harvested viral particles from well-transfected cells should have a titer of approximately 1-5 x 10^6 TU/ml when measured on NIH-3T3 or HEK293T cells.*

Functional titer and relative 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 7**.

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.

Protocol – Titering CLIP-All 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 4**.
   (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 42 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

Dilution table and schematic for titration protocol

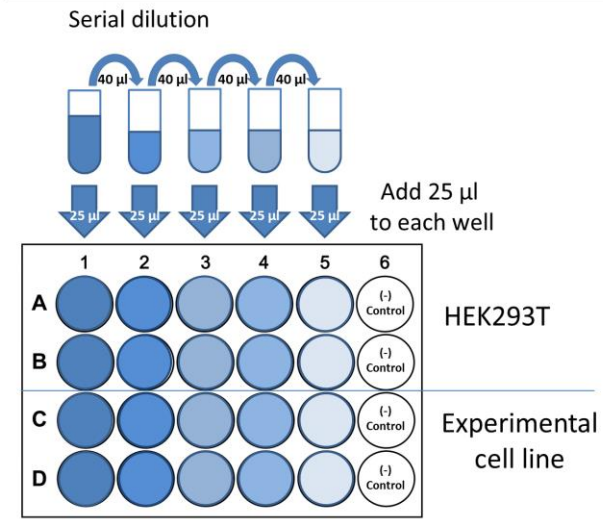


Figure 7. Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 4 Dilution factors for calculating viral titer

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

Example:
Typical virus production will yield 1-5 x 10^6. The expected number of fluorescent colonies for a viral titer of 1 x 10^6 would yield the following number of fluorescent colonies in titering assay:

Tube	1	2	3	4	5	6
Dilution	1/5	1/5	1/5	1/5	1/5	n/a
Diluted titer TU/ml	200,000	40,000	8,000	1,600	320	0
ml transduced cells	0.025	0.025	0.025	0.025	0.025	0
Fluorescent colonies expected	5,000	1,000	200	40	8	0

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

10. Selection - Enrichment for increased genome editing efficiency

After 24-48 hours, transduced cells with pCLIP-All can be selected using antibiotic resistance or fluorescent protein (FP) expression. The transEDIT Vectors/Lentiviral particles 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 (or appropriate antibiotic) 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 7** 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 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.

It may be necessary to isolate clonal populations of cells so that each cell in the culture contains the desired Cas9 expression. If cells transfected with Cas9 vectors 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.

If the pCLIP-All with an antibiotic resistance is chosen, cells can selected on the appropriate antibiotic and colonies isolated.

11. Primary Selection Screen: Recommendations & Considerations

Once functional titer, transduction efficiency and puromycin 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 (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 or less >95% of 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).

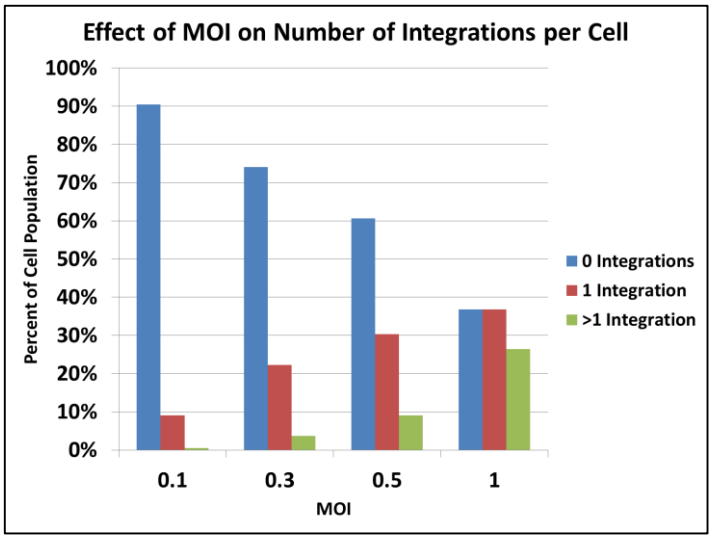


Figure 8: Higher MOIs result in a higher number of cells with multiple integrations. An MOI of 0.3 is recommended for pooled screening to limit the number of cells with multiple integrations (4%) and limit the number of cells required for transduction.

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 = 1.5×10^6

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

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 puromycin (if required)
- Number of live cells that should be plated when splitting
- Number of cells that should be harvested for each sample

Required materials

- Complete media for experimental cell line
- Complete media for experimental cell line with puromycin (if required)
- 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 puromycin).

Puromycin 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

11. 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).

    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.

12. 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 x 10^6 transduced cells. To maintain the 1000-fold coverage of gRNA used during the screen, at least 1.5 x 10^6 cells are needed for gDNA isolation from each sample.

12. 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 hairpin. Indexes are then added to each sample using the indexed primers in **Appendix 5** and the pooled PCR product from all samples may be analyzed in parallel using NGS analysis.

Individual gRNA are amplified from experimental genomic DNA with two rounds of PCR. The primary PCR amplifies the gRNA and the flanking region. The secondary PCR uses nested primers to enrich for the primary PCR amplicons using modified primers adapted for NGS on an Illumina® sequencer.

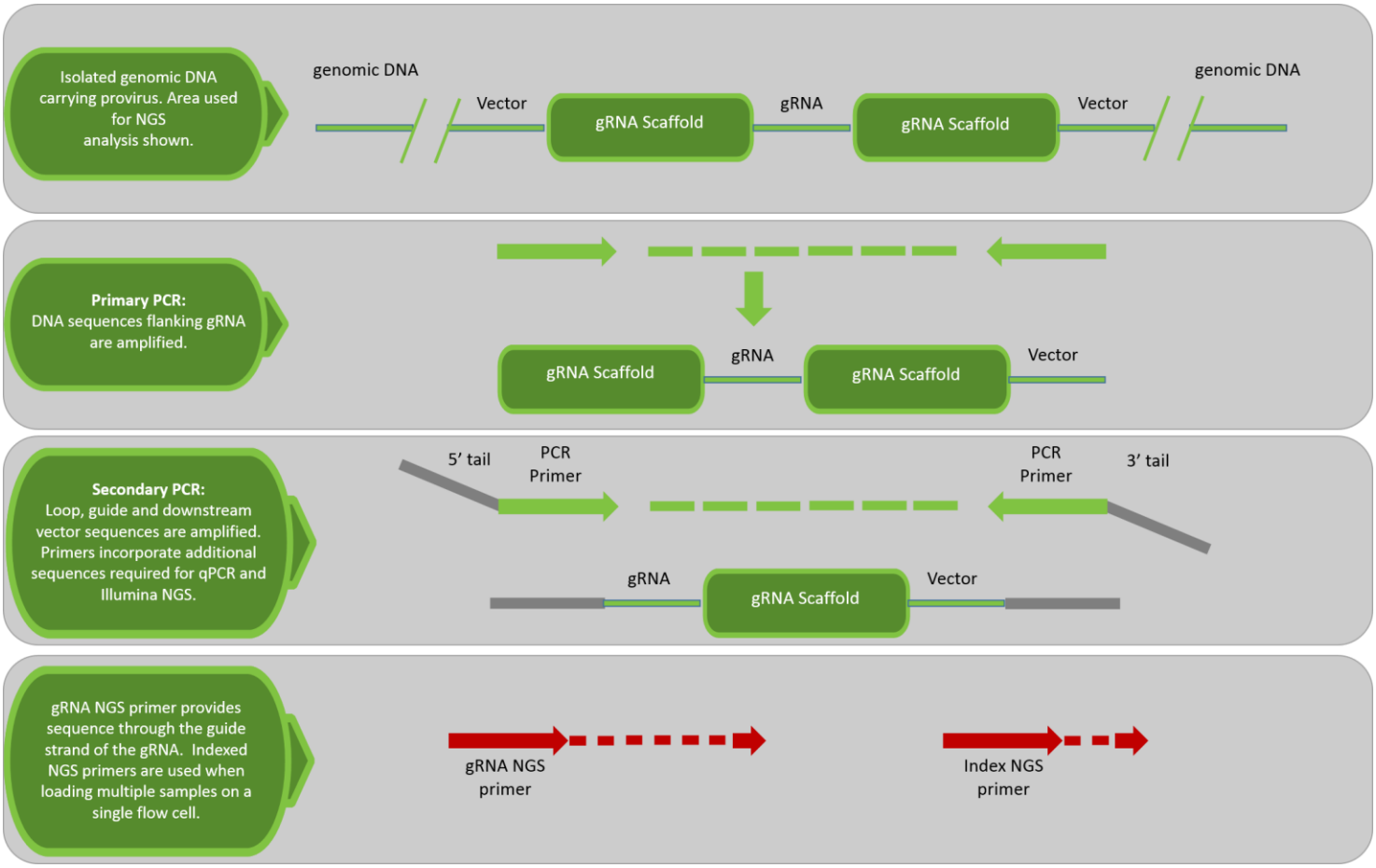


Figure 9. Schematic of PCR primers and sequencing primers as aligned with viral insert.

Primary PCR

Primary 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. The combined PCR reactions for each sample should include all extracted genomic DNA to maintain representative 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 gDNA (genomic DNA) per sample be used for amplification.

- 1500(gRNA) x 1000(coverage) x 6.6^{-12} (g/diploid genome)= 9.9^{-6} grams gDNA (or 9.9µg)

Each 50 µl PCR reaction will contain 850 ng of gDNA.

- 9.9 µg (or 9900 ng) / 850 ng DNA = 11.6 reactions

Twelve PCR reactions per sample will be required for gDNA amplification from this pool.

Required materials

- KOD Hot Start Polymerase (Novagen 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)
- PCR purification column (*QIAquick PCR Purification Kit, Qiagen Cat# 28104*)
- DNA gel extraction kit (*QIAquick Gel Extraction, Qiagen Cat# 28104)*
- Multiplex indexed primers in Appendix 5

Required equipment

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

Primary PCR Protocol

Note: KOD Hot Start Polymerase, Novagen Cat# 71086-4 is recommended for PCR.

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 below for each sample. A 96-well PCR plate is recommended.

A master mix of all components (excluding genomic DNA) should be made and then added to wells containing the same volume and concentration of genomic DNA.

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**

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
25	95	30 sec
	57	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, briefly centrifuge the 96 well plate containing PCR reactions.
6. Select at least 4 PCR reactions per sample and run 10 µl from each on an agarose gel to verify the 574 bp amplicon.
7. Pool PCR reactions from each sample and purify following manufacturer's protocol ensuring that column capacity is not exceeded (it may be necessary to use multiple columns per sample for purification). *QIAquick PCR Purification Kit, Qiagen Cat# 28104 is recommended.*
8. Quantitate purified PCR reactions using a fluorometric assay specific for double stranded DNA (dsDNA).

Secondary PCR

Note: Each sample should be amplified using a uniquely indexed (6 base pair index) Secondary PCR Reverse primer if multiple samples are run in parallel on the sequencer.

Protocol

1. 2.0 µg of primary PCR product should be amplified across 4 reactions (500 ng DNA per 50 µl PCR reaction) for each sample.
2. Set up PCR reactions using components and volumes outlined below for each sample. A 96 well plate is recommended.

A master mix of all components should be made for each Secondary PCR Reverse- Indexed primer used (excluding DNA) which can then be added to wells containing the same volume and concentration of DNA.

Component	µl per reaction
10X KOD Buffer	5
dNTP Mix	5
$MgSO_4$ (25mM)	4
Betaine (5M)	2
Secondary PCR Forward (10 µM)	7.5
Secondary PCR Reverse(10 µM)- Indexed	7.5
KOD Hot Start Polymerase	1.5
Pooled Primary PCR DNA (500 ng)	--
Nuclease-free water	--
Total Volume	**50**

3. Set up the following PCR cycling program on a thermal cycler with a heated lid.

Cycles	Temperature (°C)	Time
1	98	5 min
15	94	30 sec
	52	30 sec
	72	30 sec
1	72	5 min
1	4	Hold

4. Place samples in the thermal cycler with heated lid on and run the PCR program outlined above.
5. After cycling is complete, centrifuge the 96 well plate containing PCR reactions.
6. Pool PCR reactions from each sample.
7. Gel purify the 452 bp product following manufacturer's recommendations from the kit used. Elute in 30 µl EB (or molecular-grade water).
   We recommend using QIAquick Gel Extraction, Qiagen Cat# 28104.

Preparing NGS adapted Amplicons for sequencing

After secondary PCR and purification, it is necessary to accurately quantitate each sample prior to NGS analysis.

Protocol

1. Quantitate gRNA amplicon libraries using a fluorometric assay specific for double stranded DNA (dsDNA).
2. Determine molarity of DNA and dilute each sample to 15 nM using the calculations below.
   - *(base pairs in DNA amplicon) X (660 grams/mol/base pair) = molecular weight (MW) of DNA in sample*
   - *(DNA ng/µl) ÷ (MW of amplicons) X 10^6 = DNA nM*
   - *(DNA nM) ÷ (15) = dilution factor for 15 nM final concentration*
   - *(Dilution factor) X (Sample µl) = Total Volume of Sample and dilution buffer*

For example:
A sample with concentration of 140 ng/µl and amplicon size of 452 bp would require the following dilution factor to achieve a 15 nM final concentration:

(452 bp) x (660 grams/mol/bp) = 298320 grams/mol
(140 ng/µl) ÷ (298320 grams/mol) x 10^6 = 469.3 nM
(469.3 nM) ÷ 15 = dilution factor of 31.3
31.3 X (3 µl sample) = 93.9 µl total volume
(3 µl sample) + (90.9 µl EB) = 15 nM sample

3. Samples diluted to 15 nM, should be analyzed by qPCR to verify concentrations of Illumina® adapted amplicons. NGS qPCR kits that are designed for quantitation with Illumina® adapted libraries will work on all samples amplified with Secondary PCR primers (typically, NGS core facilities will provide this service prior to sequencing).
4. Dilute gRNA amplicon libraries to 10 nM based on qPCR quantitation.
5. Pool equimolar (uniquely indexed) amplicon libraries. It is not necessary to use the entire volume from each library when pooling, 5-10 µl from each should be sufficient.

Sequencing NGS adapted Amplicons

An NGS core facility is recommended if assistance is needed for NGS sequencing and analysis. The instructions below describe changes (to standard Illumina® NGS protocols) that are required to prepare and sequence gRNA amplicon libraries.

A 50 base pair, single read sequencing run is sufficient to sequence through the unique 20 bases of the gRNA and identify gRNA. Follow manufacturer protocols for preparing libraries (including denaturing, diluting and loading) and sequencer runs on an Illumina® sequencer. We recommend loading ~12 - 18 pM for the pooled libraries; however, optimization may be necessary to obtain cluster densities that are recommended by the manufacturer.

Note the following when using Illumina kits:

- The custom gRNA Read 1 Primer is provided as 100 µM, dilute accordingly (typically 1:200 in HT1 buffer).
- Illumina® provided Read 1 and Read 2 Primer Mixes should not be used/selected for sequencing gRNA amplicon libraries.

- PhiX control should not be run with gRNA amplicon libraries as the custom gRNA Read 1 primer is not compatible with PhiX control.
- If samples were amplified using Secondary Indexed Reverse Primers, Illumina provided Index Primer Mix should be used/selected for index reads (indexes correspond to Illumina® TruSeq indices 2, 4, 5, 6, 7, 12)

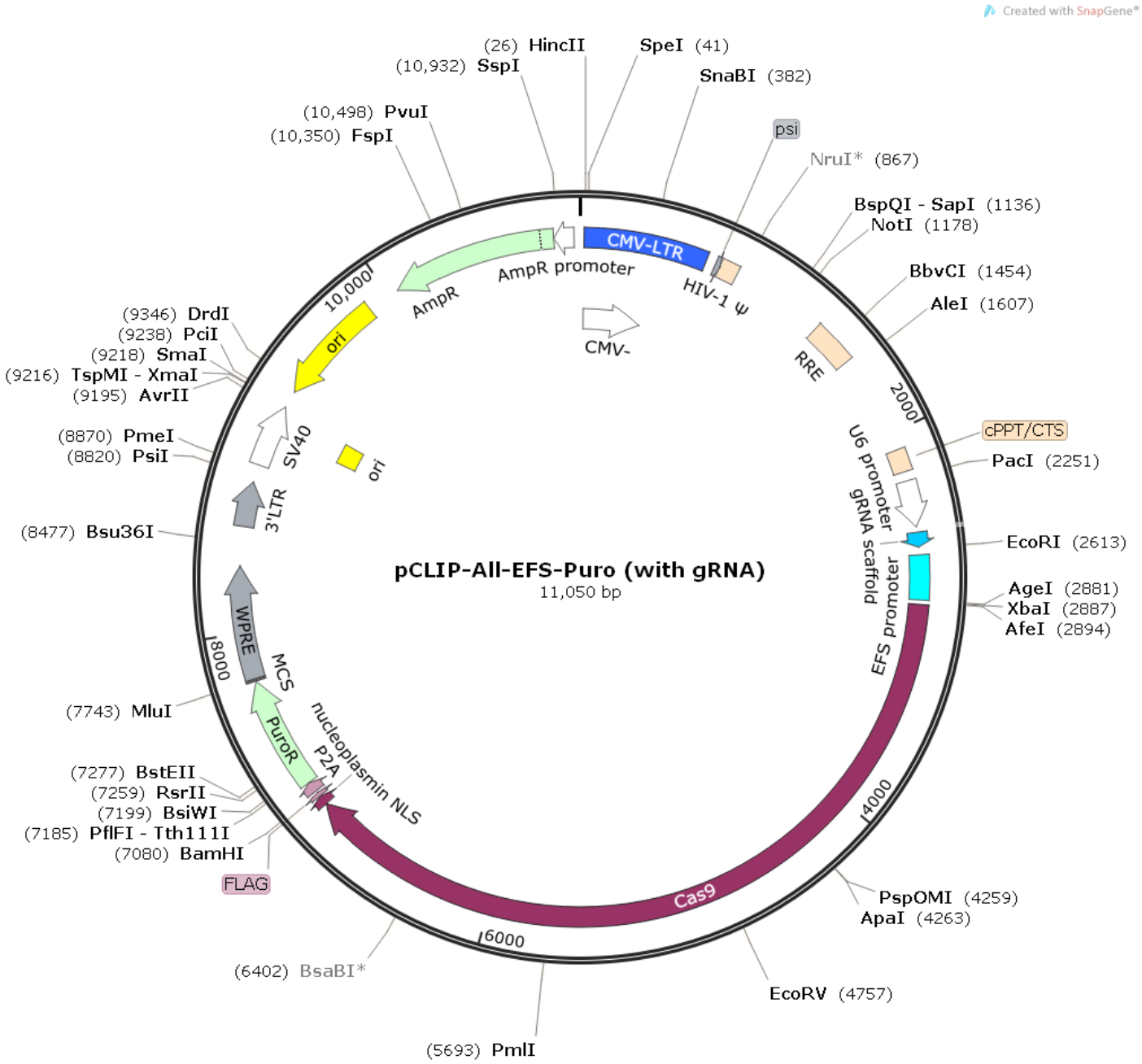
Data Analysis

The custom gRNA Read 1 Primer binds to the scaffold of the gRNA (as shown in Figure 9) therefore, the first base read is the 5' end of the gRNA sequence. The 20 bases of the gRNA sequence can be used to identify the gRNA. Multiplexed samples should be separated and analyzed according to their index reads.

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 individual 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.

13. Appendices

Appendix 1 – pCLIP-All-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	2531	2606
EFS promoter	2648	2880
Cas9	2904	7007
P2A	7089	7145
PuroR	7146	7742
MCS	7744	7749
WPRE	7756	8347
3'LTR	8552	8787
SV40	8881	9210
ori	9061	9196
AmpR	10055	10915
AmpR promoter	10916	11020

Figure 10. Detailed map of the pCLIP-All-EFS-Puro vector. The pCLIP vectors include options for additional selection markers. Visit www.transomic.com for detailed vector information for additional 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.

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

Appendix 4 - Limited use licenses

This product is covered by several limited use licenses. For updated information please refer to www.transomic.com/support/productlicenses

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Appendix 5 - PCR and NGS sequencing primer sequences

Primary NGS Forward (5'->3')
TTTTAAAAGAAAAGGGGGGATTGGGGGG

Primary NGS Reverse (5'->3')
CTGACGGGCACCGGAG

Crispr Secondary PCR Forward primer (5'->3')
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgggcctatttcccatgattcc

Secondary PCR Reverse Indexed primer (5'->3')
CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg

6 bp Index

Secondary PCR Reverse Index	Secondary PCR Primer sequences with 6 base pair index (5'->3')
CRSPR-NGS-R Index #2	5'CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg
CRSPR-NGS-R Index #4	5'CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg
CRSPR-NGS-R Index #5	5'CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg
CRSPR-NGS-R Index #6	5'CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg
CRSPR-NGS-R Index #7	5'CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg
CRSPR-NGS-R Index #12	5'CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTccacttttttcaagttgataacgg

Note: Since the sequences shown in the above table are reverse primers in 5'->3' orientation, the highlighted indexes are shown as reverse complements. Indexes 1-12 above correspond to Illumina TruSeq indexes 1-12.

gRNA Read 1 Primer (5'->3')
cgatttcttggctttatatatcttGTGGAAAGGACGAAACACCG

If low level multiplexing is needed (6 samples or less), please refer to *Multiplexing Sample preparation Guide (Illumina® Part# 1005361)* for recommendations on which sets of the indexes above can be used together.

Appendix 6 – References and recommended reading

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Genetic screens in human cells using the CRISPR-Cas9 system. Science 343, 80–84.