transEDIT™ Lentiviral gRNA (pCLIP-gRNA) Target Gene Sets with Inducible Cas9

Viral Particles

CCIHV1001, CCIMV1001, CCIRV1001

This manual provides information for the stable transduction of the transEDIT lentiviral gRNA expression vector with inducible Cas9 and TRE3G transactivator. Appendix 2 contains basic safety information for production and handling of lentiviral particles. Review local safety guidelines for complete regulations.

Cas9 must be stably transduced into cell lines, because inducible control of the TRE promoter controlling Cas9 expression is only achieved after the lentivirus integrates into the genome of the target cell. Transfection with a plasmid will allow constitutive expression. Thus, the following protocols require the use of lentiviral particles to produce a stable, inducible Cas9 cell line. Once produced, a clonal cell line optimized for Cas9 inducibility and cell vitality can be used for multiple experiments with different gRNA.

The protocols in this manual are broken out into three sections:

Section I: Plasmid propagation, kill curves, virus production and titration
Section II: Expressing gRNA in a cell line with inducible Cas9 expression.
Section III: Generating a cell line with inducible Cas9 expression.

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Introduction

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

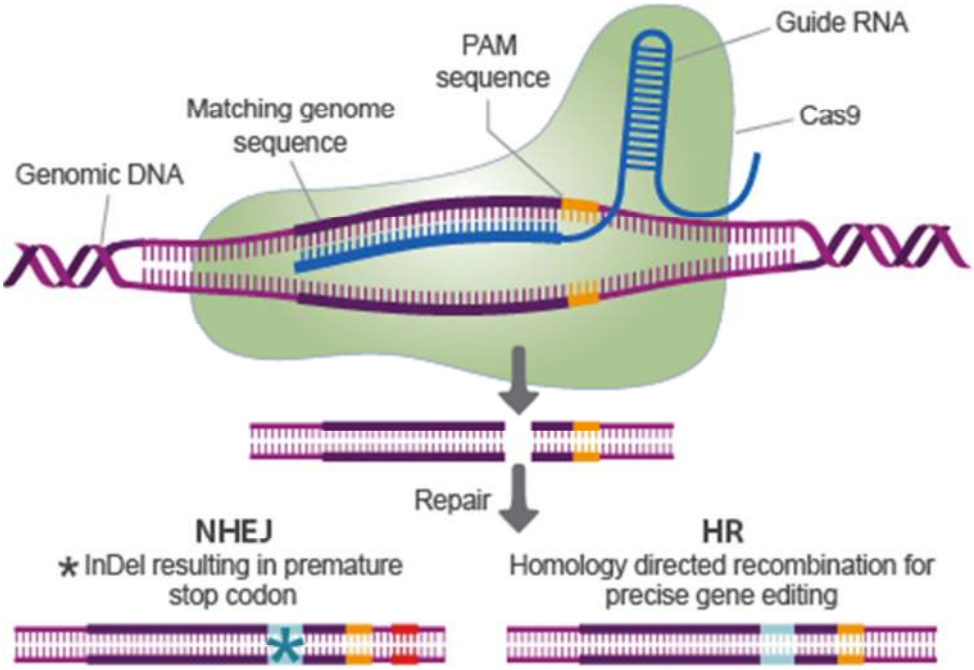


Figure 1. Schematic representation of RNA-guided double-stranded DNA cleavage by CRISPR/Cas9 using a programmable guide RNA.

Lentiviral gRNA and inducible Cas9 system overview

The pCLIP-gRNA expression vector allows transient and stable transfection; as well as the stable delivery of gRNA into host cells via a replication-incompetent lentivirus. The pCLIP-gRNA vector is designed to be used in conjunction with a Cas9 expression vector. Inducible expression of Cas9 requires two vectors in addition to the gRNA expression vectors: a Cas9 expression vector with the inducible TRE3G promoter and the constitutive transactivator (Tet-On 3G TA) expression vector.

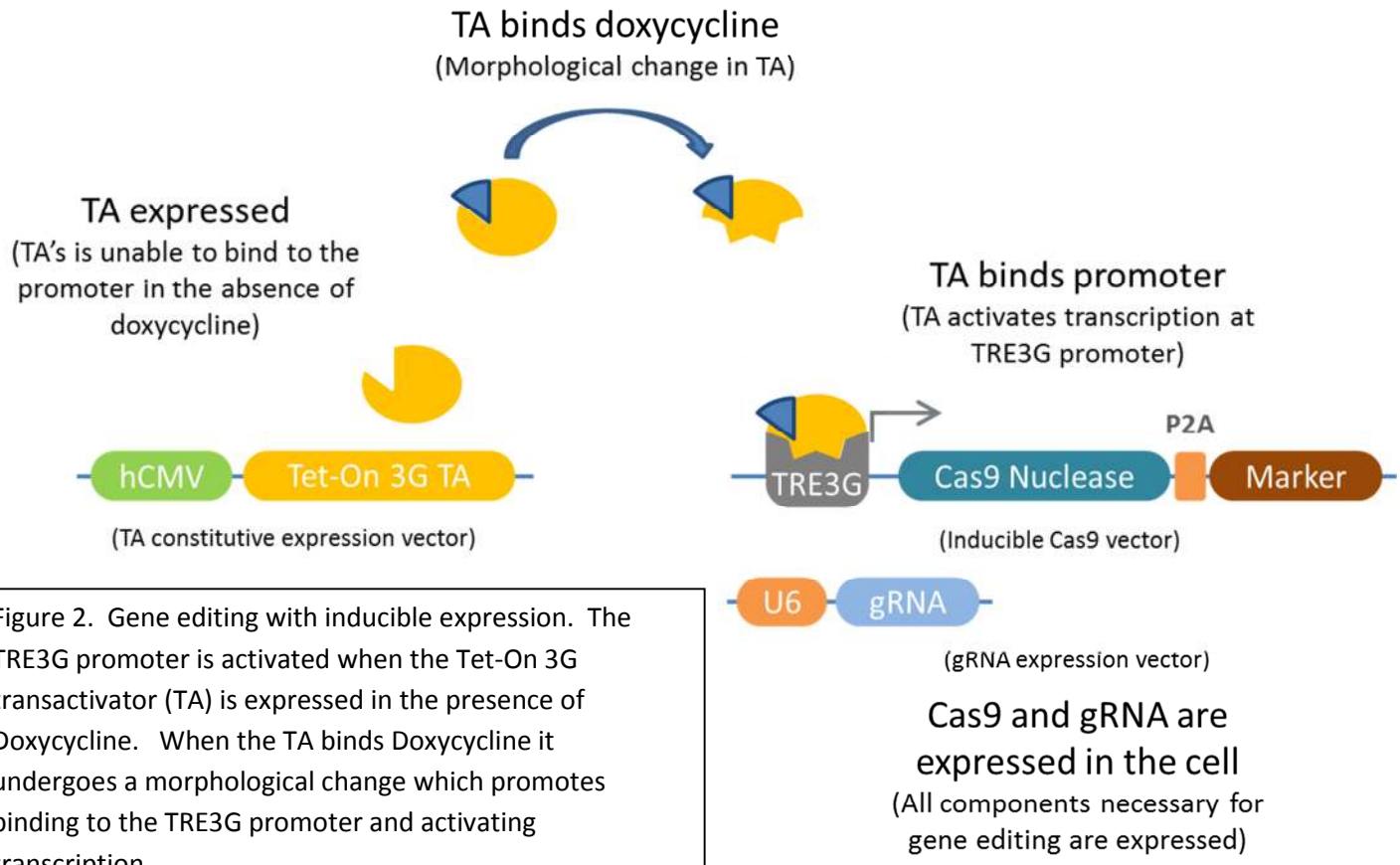
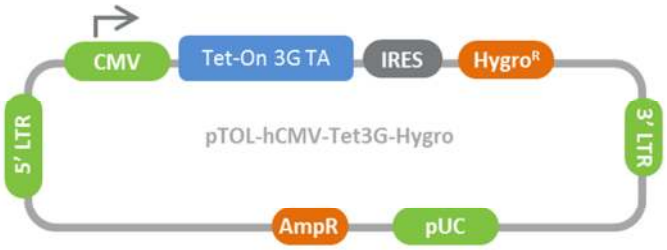


Figure 2. Gene editing with inducible expression. The TRE3G promoter is activated when the Tet-On 3G transactivator (TA) is expressed in the presence of Doxycycline. When the TA binds Doxycycline it undergoes a morphological change which promotes binding to the TRE3G promoter and activating transcription.

Vectors

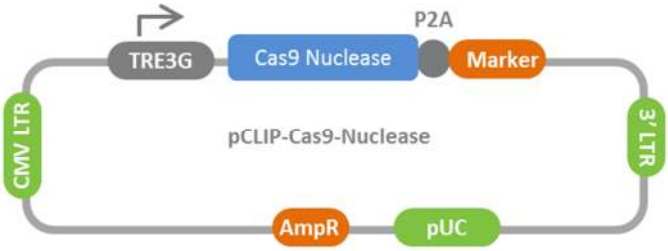
Tet-On 3G Transactivator expression vector

Expression of the Tet-On-3G transactivator (TET3G) is essential for inducible expression. The pTOL-hCMV-TET3G-Hygro is a lentiviral expression vector allowing efficient delivery and expression as plasmid or lentiviral particles. Its hygromycin selection marker makes it compatible with all markers in the gRNA expression vectors and Cas9 expression vectors.



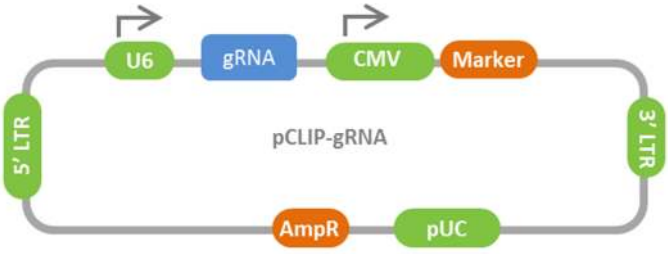
Inducible Cas9 expression vector

The TRE3G inducible promoter consists of 7 repeats of a 19 bp tet operator up stream of a minimal CMV promoter. The sequence has been optimized for very low basal expression and high maximal expression following induction (Loew *et al.*, 2010).



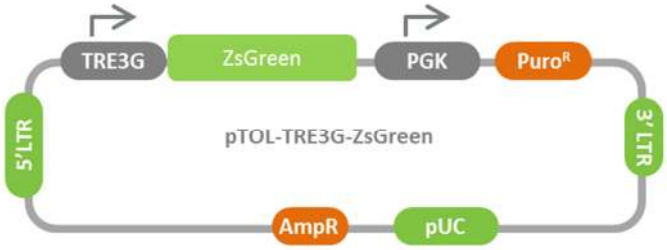
gRNA expression vector

The gRNA expression vector provides expression of the guide RNA which associates with Cas9 to direct the nuclease to the target site in the genome.



Inducible positive control

The gRNA expression vector provides expression of the guide RNA which associates with Cas9 to direct the nuclease to the target site in the genome.



General considerations for inducible expression

Doxycycline

Doxycycline (Dox), a tetracycline derivative, is bound by TET3G. Once bound, TET3G associates with the TRE3G promoter and induces transcription. The Dox concentration required for induction is far below the cytotoxic levels, and the optimized TET3G responds to even lower levels of Dox compared to previous generation tetracycline inducible systems (Zhou *et al.*, 2006). Note that the inducible system responds well to Doxycycline, and not tetracycline (Gossen & Bujard, 1995). The half-life of Dox in cell culture medium is 24 hr. Medium should be replenished every 48 hr to maintain continuous induction of Cas9 nuclease.

Tetracycline-Free Bovine Serum

Bovine serum used in cell culture media may be contaminated with tetracyclines. This can result in unwanted, elevated background expression when using Tet-On-3G system. Tetracycline-free sera are available from Clontech:

631106	Tet System Approved FBS	500 mL
631107	Tet System Approved FBS	50 mL

Detecting TET3g expression

TET3G expression can be confirmed with the TetR monoclonal antibody from Clontech.

631131	TetR Monoclonal Antibody (Clone 9G9)

I. Propagation

Materials for individual and plate replication

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

Propagate culture for storage

Lentiviral plasmid bacterial stock cultures should be propagated in LB broth with ampicillin or carbenicillin (100 µg/ml) at 30°C for 30 hours or until the culture appears turbid. 2-10 ml starter cultures can be inoculated using 2 to 10 µl of the glycerol stock provided. Once turbid, place 920 µl of culture into a polypropylene tube and add 80 µl sterile glycerol (8% glycerol). Mix well and store at –80˚C. Glycerol stocks kept at -80°C are stable indefinitely as long as freeze/thaw cycles are minimized.

II. Plasmid isolation

For transfection and transduction experiments plasmid DNA will first have to be extracted from the bacterial cells. Cultures should be grown in LB broth with ampicillin or carbenicillin (100 µg/ml) at 37°C overnight or until the culture appears turbid. 2-10 ml starter cultures can be inoculated using 2 to 10 µl of the glycerol stock provided. Either a standard plasmid mini-preparation or one that yields endotoxin free DNA can be used. When isolating plasmid DNA for virus production using endotoxin free kit will generally yield higher viral titers.

III. Selection kill curve

The optimal antibiotic concentration should be determined for a cell line prior to transduction or transfection. The pCLIP-gRNA and pCLIP-Cas9 Nuclease vector have an option for puromycin or blasticidin selection in mammalian cells. The pTOL-hCMV-TET3G-hygro vector has hygromycin resistance. These can be used to co-select cells for expression of both plasmids. To establish stable cell lines, once transfection/transduction has occurred, the cells can be treated to select for stable integrants. Since cell lines differ in their sensitivity to antibiotics, the optimal concentration (pre-transfection/transduction) should be determined. In the following protocol the lowest concentration that provides adequate selection is determined for the experimental cell line.

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

Required materials

- Complete media experimental cell line
- Puromycin (1.25 µg/µl stock solution)
- Blasticidin (1.25 µg/µl stock solution)
- Hygromycin (50 mg/ml stock solution)
- 24-well tissue culture plate

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C

Protocol

1. Plate 5 x 10^4 cells per well in 11 wells of a 24-well tissue culture plate using media without puromycin.

Prepare dilutions in culture media for antibiotic titration as shown in Table 1. Use a puromycin and blasticidin stock solution of 1.25 µg/µl stock solution and use a hygromycin stock solution of a 50 mg/ml.

Table 1 Volumes of stock solutions required for establishing optimal antibiotic concentration

Volume of Puromycin* Stock Solution (µl) per 500 µl media	Final Concentration (µg/ml)	Volume of Hygromycin Stock Solution (µl) per 500 µl media	Final Concentration (µg/ml)
0	0	0	0
0.2	0.5	1	50
0.4	1	2	100
0.6	1.5	3	150
0.8	2	4	200
1	2.5	6	300
1.2	3	8	400
1.6	4	10	500
2	5	12	600
3	7.5	16	800
4	10	20	1000

*Use the same concentrations for blasticidin as for puromycin.

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 concentration will kill the cells rapidly (2 - 4 days).

IV. 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 protocol. Selection may be used to remove untransduced cells. A kill curve should be performed as described in a previously.

Note: An alternate protocol for titering Cas9 expressing viral particles follows the standard titering protocol. Inducible Cas9 requires a cell line expressing the TET3G transactivator for titering. The expression of the fluorescent and selection markers requires induction of Cas9 for expression. The protocol for creating this cell line is outlined in detail Section II. In addition, due to the large size of the Cas9 gene, the vector packages less efficiently than the TRE3G vector or the gRNA vector. Expect 10^4 – 10^5 TU/ml.

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.

Note:

- 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 protocol 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.
- Inducible Cas9 expressing vectors require a cell line expressing TET3G. The protocol for creating this cell line is provided in Section II.

Required materials

- HEK293T cells
- Complete media for HEK293T cells and experimental cell line
- Serum free media for each cell line
- 24-well tissue culture plate
- Lentiviral particles (Harvested or purchased)
- Microcentrifuge tubes
- Polybrene
- Dox

Equipment

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

Protocol – Titering CLIP-gRNA vectors and the TOL-TET3G-Hygro transactivator expressing vector

1. Plate cells (HEK293T and experimental cell line) 24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 12 wells with complete media. Incubate for 24 hours with 5% CO_2 at 37°C.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 2 and Figure 3. (Follow the alternate protocol when titering Cas9 expressing vectors.)
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 160 µl of serum free medium to each tube containing 5-8 µg/ml Polybrene.
    c. Add 40 µl of viral stock to the first microfuge tube.
    d. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    e. Transfer 40 µl from the first microcentrifuge tube to the second tube. Mix well and discard the tip.
    f. Repeat the procedure for the 4 remaining tubes.
    g. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 200 µl of serum free media to each well containing cells.
5. Add 25 µl from each viral dilution to two wells (225 µl final volume) for a total of 10 wells per cell line. The remaining two wells (without viral particles) should be evaluated as negative controls.
6. Rock plate gently a few times to mix.
7. Incubate overnight with 5% CO_2 at 37°C.
8. Colony counting: (*Note: Counting 50-200 colonies in a well is sufficient to provide accurate titers.*)
    a. Puromycin or blasticidin titering by selection and colony counting:
        i. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
        ii. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
        iii. Use a microscope to count the number of surviving colonies.
    b. Fluorescent colony counting
        i. Replace the viral supernatant with complete media including Dox and allow induction and growth for 48 hours.
        ii. Count the number of colonies expressing ZsGreen or tRFP. A colony consisting of multiple cells should be counted as a single transduction event.
9. Use the calculation below and Table 2 to determine functional titer.
   (An alternate method for calculating titers via FACS is described in Appendix 3.)

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

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

Alternate Titering Protocol for Inducible Cas9 Vectors

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

1. Plate cells (HEK293T and experimental cell line) 24 hours prior to transduction in a 24 well plate. Plate at a density of 7 x 10^4 cells per well in 12 wells with complete media. Incubate for 24 hours with 5% CO_2 at 37°C.
2. Prepare a serial dilution series with serum free media and viral supernatant as shown in Table 3 and Figure 4.
    a. Set up 5 sterile microcentrifuge tubes.
    b. Add 1.25 ml of viral supernatant to the first tube.
    c. Add 1 ml serum free medium with 5-8 µg/ml Polybrene to each of the remaining tubes.
    d. Transfer 250 µl from the tube 1 to tube 2.
    e. Mix well by gently pipetting up and down (10 - 15 times) without creating bubbles and discard the tip.
    f. Repeat the procedure for tubes 3-5 leaving tube 6 with no viral particles.
    g. Incubate at room temperature for 10-15 minutes.
3. Remove media from each well.
4. Add 250 µl from each viral dilution to two wells for a total of 10 wells per cell line. The remaining two wells (without viral particles) should be evaluated as negative controls.
5. Rock plate gently a few times to mix.
6. Incubate overnight with 5% CO_2 at 37°C.
7. Replace media with media containing Dox to induce marker expression.
8. Colony counting: (*Note: Counting 50-200 colonies in a well is sufficient to provide accurate titers.*)
    a. Puromycin or blasticidin titering by selection and colony counting:
        i. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
        ii. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
        iii. Use a microscope to count the number of surviving colonies.
    b. Fluorescent colony counting
        i. Replace the viral supernatant with complete media including Dox and allow induction and growth for 48 hours.
        ii. Count the number of colonies expressing ZsGreen or tRFP. A colony consisting of multiple cells should be counted as a single transduction event.
9. Use the calculation below and Table 3 to determine functional titer.
   (An alternate method for calculating titers via FACS is described in Appendix 3.)

Standard dilution table and schematic for titration protocol

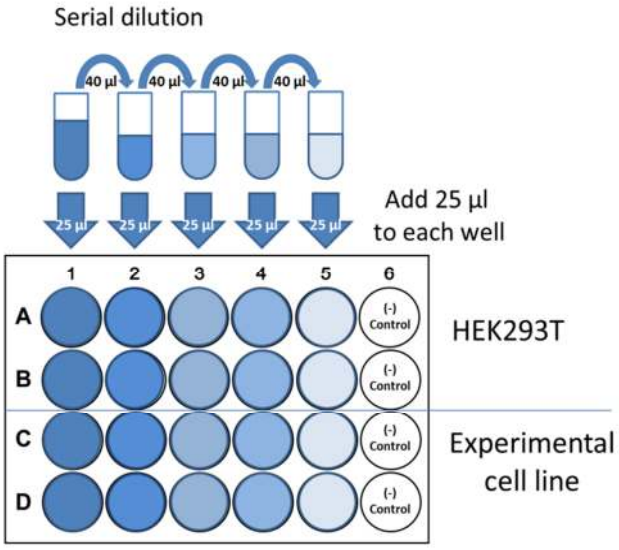


Figure 3 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 2 Dilution factors for calculating viral titer

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

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

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

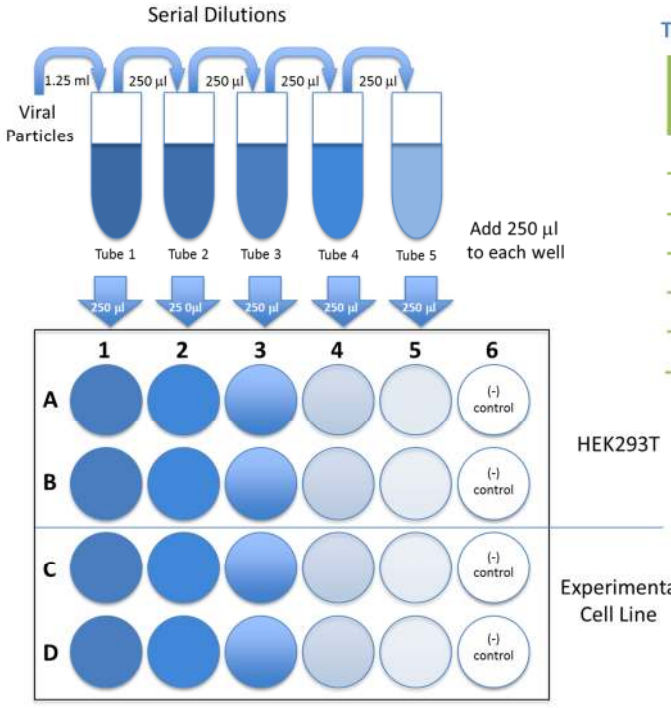


Figure 4 Schematic of serial dilution for viral particle titering. (-) indicates untransduced control.

Table 3 Dilution factors for calculating viral titer

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

*For Cas9 expressing vectors start with undiluted

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

Section II

Expressing gRNA in a cell line with inducible Cas9 expression

An inducible Cas9 cell line can be transduced with lentiviral particles expressing gRNA to induce gene editing. The following protocol covers transduction, induction and isolation of clonal cell lines. The optimal Dox concentration should be determined prior to transduction with the CLIP-gRNA vector. Cells expressing Cas9 with a fluorescent marker can be optimized for induction using the marker as an indicator of expression level.

Levels of gene editing may vary between cell lines and gRNA designs. The percent of alleles with Indels can be determined through assays such as the Surveyor assay (not covered in this protocol). The percentage should increase over time making it more likely to isolate clonal lines with the desired deletion. Single cell clones can be isolated and characterized 5-11 days after induction. Longer inductions should result in a larger percent of the population with the desired knock out, but may result in a higher risk of offtarget effects. Clonal populations can be isolated to characterize individual mutants.

I. Transduction with gRNA expression vectors

This protocol provides a basic outline of the transduction process. The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml antibiotic
- Dox concentration

Required Materials

- Experimental cells
- HEK293T cells
- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with puromycin or blasticidin
- Transduction media containing viral particles (optimized for serum and Polybrene concentration)
- Selection media
- Induction media
- Lentiviral Particles
- Antibiotic (concentration determined empirically by kill curve)
- Sterile microcentrifuge tubes
- Polybrene
- Dox

Equipment

- Automatic pipettor /Pipette-aid (for tissue culture)
- Pipettor (for handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO^2 cell culture incubator at 37°C

Protocol:

Prepare cells

1. Plate cells such that they are actively dividing and 60 - 70% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction. The volume of media per well should be the minimum volume that the cells can will be maintained without drying out.

Transduce cells

4. Exchange media with transduction media.
   (Note: media should be serum free for maximum transduction efficiency. Alternatively, see the titering protocol in Section I for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with induction media (includes Dox with no selection reagent).

Selection

7. Allow cells to grow for 48 hours.
8. Select for transduced cell.
   a. Vectors expressing gRNA with an antibiotic resistance marker
      i. Replace media with selection media.
      ii. Continue feeding cells selection media until untransduced cells have been removed.
   b. Vectors expressing gRNA with a fluorescent marker
      i. Cells may be sorted via FACS analysis.
      ii. Plate cells expressing the fluorescent markers. Cells with greater fluorescence tend to have a higher gene editing efficiency.

Single-Cell Cloning

A number of methods are available for single cell cloning. Some cell lines may perform better using a specific method. The following outline the process for dilution cloning.

Clonal populations can be generated manually by simply diluting the cells and plating 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 3-5 96-well plates be plated for each number of cells in the range as approximately half of the well should be empty. 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 likely to be derived from a single, clonal cell.

II. Enrichment for increased genome editing efficiency (Optional)

Prior to clonal isolation the population of cells with high gene editing efficiency can be selected for allowing shorter induction times by using antibiotic resistance or fluorescent protein (FP) expression. The transEDIT pCLIP-Cas9 Nuclease Lentiviral particles co-express several different selectable markers via a 2A "self-cleaving" peptide, including blasticidin resistance (Blast^R) and puromycin resistance (Puro^R) genes, as well as ZsGreen, and turboRFP (tRFP) FPs. Expression of Cas9 and the marker must be induced prior to selection.

Antibiotic selection:
Refer to the protocol for the kill curve in Section I 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. Induce expression using the optimal concentration of Dox determined in Section III.
3. Begin the antibiotic selection by replacing the medium with complete medium supplemented with puromycin or blasticidin. Use concentrations higher than the optimal one determined in the kill curve. Multiple concentration may be tested to ensure some cells survive.
4. Replace the selective media every 2-3 days. Monitor the cells daily and observe the percentage of surviving cells.
5. Collect samples for assay.

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

III. Turning off expression

Note: Dox adheres to many cells and culture plates. Turning expression off after induction requires cell to be split, rinsed and transferred to a new plate or well that has not been exposed to Dox.

Protocol

1. Wash the cells in PBS.
2. Split cells and transfer to new plate using Dox-free media.
3. After splitting the cells into fresh media without Dox, incubate for 3 hours.
4. When cell are adherent, rinse cell with PBS three times.
5. Continue to feed cells with Dox-free media.

Note: The induced protein will dissipate within 72 hours. Do not add selection media unless the cells are induced again.

A cell line with inducible Cas9 expression is the basis for inducible gene editing and limits potential offtarget effects that could occur from long-term constitutive expression. Once created, multiple gRNA may be applied. The process of generating a cell line with inducible Cas9 requires 3 main steps:

1. Transduction of the target cell line with lentiviral particles expressing the TET3G Tet-On transactivator (TET3G).
2. Isolation of clonal cell lines to select one with optimal inducibility and viability.
3. Transduction of TET3G expressing line with a lentiviral vector expressing Cas9 under the control of the TRE3G promoter.

The resulting cell line will have the complete system for inducing Cas9 expression with the addition of Doxycyline (Dox) to the media. Inducing expression of Cas9 while a gRNA is expressed allows CRISPR/Cas9-based gene editing.

I. Generating TET3G-Expressing Cell Lines

To regulate Cas9 expression from the TRE3G promoter, cells must express the TET3G. The lentiviral-based pTOL-TET3G-Hygro expression vector allows for rapid generation of TET3G-expressing cells through transduction with viral particles. Isolating clonal cell populations is recommended to limit variation in results when passaging the cell line. The transduction protocol is followed by a protocol for isolating single cells populations.

It is critical that cells express relatively high levels of TET3G in order to induce sufficient levels of Cas9 nuclease. Low levels of Cas9 often result in poor gene editing efficiency. However, high TET3G expression may result in the loss of controlled Cas9 expression leading to unacceptable levels of leakiness (non-induced Cas9 expression). A protocol for selecting TET3G-expressing cells that exhibit the appropriate balance of highly induced Cas9 expression with little to no leakiness is provided.

Required Materials

- Experimental cells
- HEK293T cells
- Complete media for experimental cell line and HEK293T cells
- Serum free media for each cell line
- 24-well tissue culture plate
- 6-well tissue culture plate
- Lentiviral Particles
- Antibiotic (concentration determined empirically by kill curve)
- Sterile microcentrifuge tubes
- Polybrene

Equipment

- Automatic pipettor /Pipette-aid (for tissue culture)
- Pipettor (for handling of viral particles)
- Disposable or autoclaved tissue culture pipettes
- CO^2 cell culture incubator at 37°C

Transduction Protocol

The following describes a protocol for stable integration/expression of TET3G into the experimental target cell line by transduction with pTOL-TET3G-Hygro Lentiviral Particles. It is recommended that the protocol be performed in parallel using HEK293T cells as a positive control.

Due to differences in transduction efficiency and vector expression between cell lines, the protocol provides for transduction of cells at two multiplicity of infections (MOIs); low (MOI = 1 to 2) and high (MOI = 5). The MOI is the number of transducing units per cell in a culture. Transduction at two MOIs ensures that cells expressing a wide range of TET3G levels are generated. Sufficient viral particles must be produced to transduce two cell lines (*e.g.* experimental cell and HEK293T) at both MOIs.

The following protocol represents the standard procedure for generating HEK293T cells (positive control) expressing TET3G. Optimal cell numbers, serum and polybrene concentrations, times, antibiotic concentrations, and culture conditions are likely to be different for the experimental cell line.

The following should be optimized prior to transduction:

- Transduction media: % Serum, Polybrene µg/ml
- Time exposed to transduction media: hours or overnight
- Selection media: µg/ml antibiotic

Required materials

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

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment

Protocol:

Prepare cells

1. Plate cells in 6 wells of a 24-well plate such that they are actively dividing and 60 - 70% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction.
    a. The volume of media per well should be the minimum volume that the cells can will be maintained without drying out.
    b. Prepare sufficient media to transduce 2 wells at an MOI of 1 and 2 wells at an MOI of 5.

Transduce cells

4. Exchange media with transduction media.
   (Note: media should be serum free for maximum transduction efficiency. Alternatively, see the titering protocol in Section I for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with complete media (no selection reagent).

Antibiotic selection and expansion

7. Allow cells to grow for 48 hours.
8. Replace media with selection media.
9. Continue feeding cells selection media until untransduced cells have been removed.
10. Trypsinize cells from each of the wells and re-seed into a 6-well plate containing 2 ml of complete medium supplemented with hygromycin at the concentration determined by the kill curve.
11. Replenish selection media containing every two days until cells in the 6-well plate grow to near confluency.
12. Expand cultures from each well while maintaining hygromycin selection and cryopreserve cells appropriately.

The cells are a pooled, heterogeneous population with a wide range of TET3G expression levels depending on the number of lentiviral vector integrations and the locations in the host cell genome where the vector integrated. Single cell isolates will be collected from each to create a clonal cell line that can be transduced with a vector expressing Cas9 under the TRE3G promoter recapitulating the complete Doxycycline-based inducible Cas9 expression system.

Single-Cell Cloning

A number of methods are available for single cell cloning. Some cell lines may perform better using a specific method. The following outline the process for dilution cloning.

Clonal populations can be generated manually by simply diluting the cells and plating 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 containing the appropriate concentration of hygromycin. It is recommended that 2-3 96-well plates be plated for each of the starting pools of cells created in the previous section. A majority of the wells will be empty or have more than one colony. If using adherent cells, cultures growing out from one colony can be identified by microscopy and expanded. The HEK cells serve as a positive control as these tend to grow rapidly from single cells.

A minimum of 10 clonal cell lines should be isolated from each starting population of cells and tested for levels of induction and leakiness.

II. Testing TET3G Clonal Cell lines

A lentiviral vector expressing ZsGreen under the control of the TRE3G promoter (pTRE3G-ZsGreen) is used to assess inducibility via fluorescence. Cells are compared to the parent cell line to determine changes in growth rate or viability that have occurred in the clonal cell lines.

1. Plate clonal cell lines in a 96-well plate as shown below.
2. Transduce cells with TRE3G-ZsGreen Lentiviral Particles following plate format below. We recommend an MOI of >1.
3. Allow the cells to recover for 24 hours. Add various concentrations of Dox following format below in figure 5.
4. 48 hours after the addition of Dox assess the cells:
    a. Cell vitality can be assessed by comparison of the "No virus/No Dox" samples to the parent cell line.
    b. Maximal induction can be determined qualitatively using a fluorescent microscope. The brightest fluorescence is expected to correspond with the highest expression of Cas9 in the final cell line.
    c. Minimal leakiness can be determined by comparing the cell lines transduced with the inducible fluorescent vector but without Dox ("Viral particles with no Dox").
5. Choose cell line exhibiting highest induction, little to no leakiness, and viability similar to experimental cell line (No Tet3G).

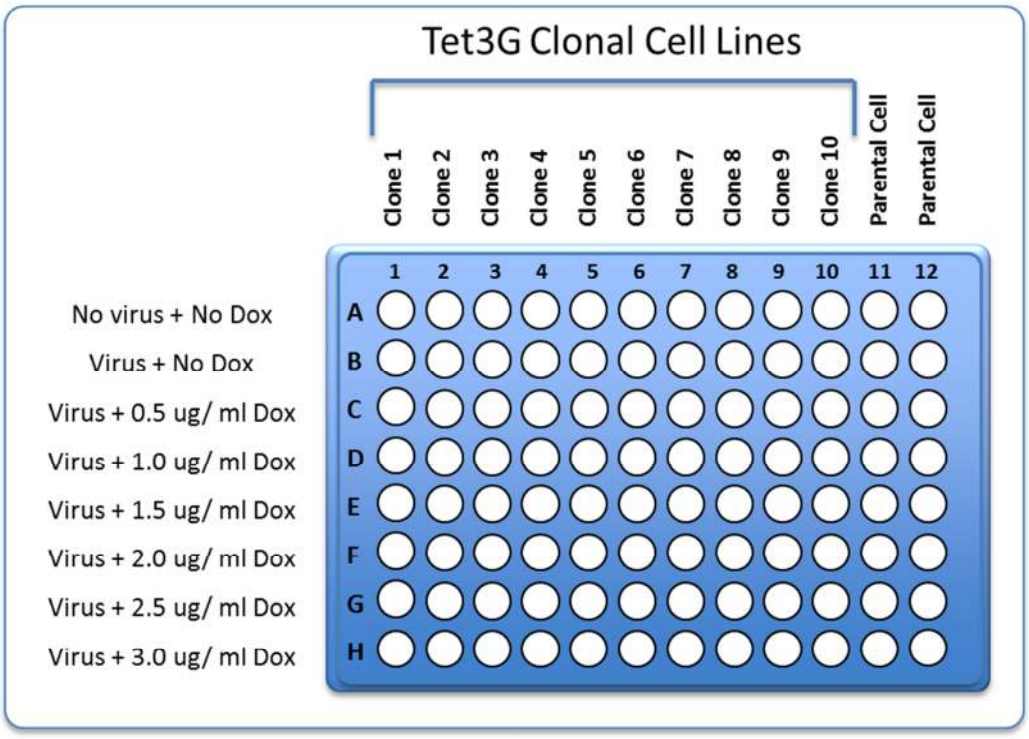


Figure 5. Diagram of 96-well plate with a range of Dox concentrations used to select the cell line with the greatest induction and least amount of background by comparing the greatest fluorescence shown in the Dox treated well to the fluorescence in the "no Dox" wells. The vitality can be determined by comparing the different clone isolates to the parental cell line plated in the far right column.

III. Transduction with inducible Cas9

Considerations for optimizing transduction and selection of TET3G-expressing cell line

After the TET3G-expressing cell line has been generated and tested, the next step is to introduce the inducible Cas9 expression cassette. This is done by transducing the TET3G-expressing cells with transEDIT pCLIP-Cas9 Nuclease-TRE3G lentiviral particles followed by selection.

Required materials

- Complete media for experimental cell line
- Selection media: complete media for experimental cell line supplemented with puromycin or blasticidin
- Transduction media containing viral particles (optimized for serum and Polybrene concentration)
- Induction media containing Dox at the optimal concentration.

Equipment

- Automatic pipetter /Pipette-aid
- Disposable or autoclaved tissue culture pipettes
- CO_2 cell culture incubator at 37°C
- Assay specific equipment

Prepare cells

1. Plate cells such that they are actively dividing and 60 - 70% confluent at the time of transduction.
2. Feed cells with complete media 3 - 4 hours prior to transduction.
3. Make transduction media just prior to transduction. The volume of media per well should be the minimum volume that the cells can will be maintained without drying out.

Transduce cells

4. Exchange media with transduction media.
   (Note: media should be serum free for maximum transduction efficiency. Alternatively, see the titering protocol in Section I for information on transduction optimization.)
5. Incubate cells 12 - 24 hours in transduction media.
6. Replace transduction media with induction media (includes Dox with no selection reagent).

Selection

7. Allow cells to grow for 48 hours.
8. Select for transduced cell.
    a. Vectors expressing Cas9 and an antibiotic resistance marker
        i. Replace media with selection media.
        ii. Continue feeding cells selection media until untransduced cells have been removed.
    b. Vectors expressing Cas9 and a fluorescent marker
        i. Cells may be sorted via FACS analysis.
        ii. Plate cells expressing the fluorescent markers. Cells with greater fluorescence tend to have a higher gene editing efficiency.

Single-Cell Cloning - Generation of inducible Cas9 cell line

Clonal populations can be generated in a similar manner to clonal isolates of the TRE3G expressing cells through dilution plating. However, the fluorescent and selection markers must be induced with Cas9 prior to selection. So, the cells are not selected during single cell isolation.

Dilute cells and seed from 0.5 to 2.0 cells per well in a total volume of 100 µl/well of complete culture medium (no selection reagent added). 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 likely to be derived from a single, clonal cell.

Cell inducibility and vitality should be tested following Cas9 induction. Check isolates for their markers following induction.

Appendices

Appendix 1 – Detailed vector information

Detailed vector maps and sequence can be obtained on the Documents page for CRISPR on transOMIC.com.

Appendix 2 – Safety and handling of lentiviral particles

Recombinant lentivirus is considered a Biosafety Level 2 organism by the National Institutes of Health and the Center for Disease Control and Prevention. However, local health and safety regulations should be determined for each institution.

For more information on Biosafety Level 2 agents and practices, download Biosafety in Microbiological and Biomedical Laboratories (BMBL), Fifth Edition (Revised December 2009) published by the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and NIH. The publication can be found here: http://www.cdc.gov/biosafety/publications/bmbl5/.

If additional measures are needed, review biosafety guidance documents such as the NIH's "Biosafety Considerations for Research with Lentiviral Vectors" which refers to "enhanced BL2 containment". More information can be found through the NIH Office of Biotechnology Activities web site (http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html)

Summary of Biosafety Level 2 Practices

The following is meant to be a summary of Biosafety Level 2 practices and should not be considered comprehensive. A full account of required practices should be determined for each institute and/or department.

Standard microbiological practices

- Limit access to work area
- Post biohazard warning signs
- Minimize production of aerosols
- Decontaminate potentially infectious wastes before disposal
- Use precautions with sharps (e.g., syringes, blades)
- Review biosafety manual defining any needed waste decontamination or medical surveillance policies

Safety equipment

- Biological Safety Cabinet, preferably a Class II BSC/laminar flow hood (with a HEPA microfilter) used for all manipulations of agents that cause splashes or aerosols of infectious materials; exhaust air is not recirculated
- Protective personal equipment includes: protective laboratory coats, gloves, face protection if needed

Facilities

- Autoclave available for waste decontamination
- Chemical disinfectants available for spills

Appendix 3 - Alternate methods for titering

1. Puromycin or blasticidin titering by selection and colony counting:
    a. Begin the antibiotic selection by replacing the media with complete media supplemented using the optimal concentration determined in "kill curve"
    b. Continue feeding and observe the cells for approximately 7 days until you see single colonies surviving the selection. The negative control should have no surviving cells.
    c. Use a microscope to count the number of surviving colonies.
    d. Calculate the functional titer using the number of colonies visible at the largest dilution that has colonies.

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

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

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

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