

Protocol • CRISPR Genome Editing In Cell Lines

Protocol 1: Gene knockout without donor

Notes:

1. sgRNA validation: GeneCopoeia recommends that, before beginning your knockout project, you validate the function of your sgRNAs using the [IndelCheck™ Insertion or Deletion Detection system](#) (cat. # IC001/IC002), which is an assay system using the T7 Endonuclease I method. The IndeCheck™ system will also be used for screening for the presence of positive clones later in this Protocol.
2. Gene copy number: GeneCopoeia recommends determining the copy number of your target gene before you begin your project, if this information is not known. Many immortalized cell lines, especially cancer cell lines, are not diploid, and so can have 3 or more copies of a chromosome. Use GeneCopoeia's VividFISH™ FISH probes (http://www.genecopoeia.com/product/fish_probes/) to determine the number of copies of the chromosome your gene is located on.
3. Delivery method. Decide whether you are going to use lentivirus or transfection to deliver your CRISPR reagents. If you are going to use transfection, note that this procedure is optimized for use with GeneCopoeia's EndoFectin™ Max transfection reagent (cat. # FM1004-01/02; <http://www.genecopoeia.com/product/endofectin/>). For other transfection reagents, follow the instructions recommended by the manufacturer. If you are using lentivirus, this protocol begins with packaged, ready-to-use virus.
4. GeneCopoeia recommends that you do not use antibiotic selection for the Cas9- and/or sgRNA-containing plasmids, unless the transfection efficiency of your cell line is very low (<50%). Using antibiotic selection will select for cells that have sustained random integration of the plasmid. If your transfection efficiency is low, you might also be able to use FACS sorting for a fluorescent reporter in place of antibiotic selection to enrich for transfected cells, if one is present on the plasmid.
5. Quality of plasmid: It is critical to use endotoxin-free plasmid DNA of the highest quality. Determine the DNA concentration by reading the absorption at 260 nm. DNA purity is measured by using the 260 nm / 280 nm ratio (the ratio should be in the range of 1.8 to 2.0). If possible, check the plasmid integrity by agarose gel electrophoresis.
6. Condition of cells: Always use high-quality cells that are well maintained and routinely authenticated, which includes testing for bacteria, fungi, or Mycoplasma contamination. If the cells are from a recent liquid nitrogen stock, passage the cells at least 2 times before transfection.

Delivery Option A. Transfection

Before Transfection

1. 1 day prior to transfection, seed approximately 1.0×10^5 - 3.0×10^5 cells in each well of a 6-well plate containing 2 mL of complete growth medium.
2. Grow cells overnight to approximately 90%-95% confluence.

Transfection

1. Equilibrate DNA, EndoFectin™ Max reagent, and Opti-MEM® I Reduced Serum Medium (Life Technologies. **Catalog number:** 31985-088) to room temperature
2. Dilute 2.5 µg plasmid DNA with 125 µL Opti-MEM® I.
3. Incubate the mixture for 5 minutes at room temperature. Once the transfection reagent is diluted, combine it with the DNA within 30 min.
4. Combine the diluted DNA with the diluted transfection reagent. Incubate at room temperature for 5 to 20 min to allow DNA-transfection reagent complexes to form.
5. Add the DNA-transfection reagent complexes directly to the well and mix gently by rocking the plate back and forth.
6. Incubate the cells at 37°C in a CO₂ incubator for a total of 24-48.

Tech Notes:

- 1) Since transfection efficiencies vary across different cell lines, we recommend optimizing the input for best results.
- 2) For optimal results, we recommend complexing DNA with transfection reagent in serum- and antibiotic-free media and cells growing in complete media (e.g. DMEM/F12+10% FBS w/o antibiotics).
- 3) For hard-to-transfect cells (e.g. primary, stem, hematopoietic), it may be advisable to utilize a non-passive transfection method. Please follow recommended guidelines provided by the manufacturer for the specific cell type(s) being transfected.

Now you can skip to the section below entitled “Single clone isolation.”

Delivery Option B. Lentiviral transduction

Note: This protocol is intended to be a general guideline only. Different cell lines will have different requirements for growth media composition and multiplicity of infection (MOI). Where applicable, use the conditions that are optimal for your particular cell line.

As mentioned earlier, this protocol begins with virus that is already packaged and ready to use. If you need a protocol for packaging lentivirus, refer to GeneCopoeia's [Lenti-Pac™ Lentiviral Packaging Systems manual](#).

Getting Started

Storage of lentivirus

Upon receipt of a new lentivirus preparation, place the tubes in storage at -80 °C immediately. Avoid repeated freeze-thaw cycles, which can result in a loss of infectivity. If necessary, tubes of Lentifect™ lentivirus can be divided into smaller aliquots upon first thawing and placed at -80 °C.

Condition of cells

Always use healthy cells that are well maintained and passaged regularly. Make sure the culture is free from bacteria, fungi, or *Mycoplasma* contamination. If the cells are from a recent liquid nitrogen stock, passage the cells at least 2 times before transfection.

Multiplicity of infection

Before transducing with Lentifect™ lentivirus, it is essential to know the MOI for your cell line, which is the optimal number of virus particles to infect each cell with. The optimal MOI is known for many cell lines. Refer to the table below for a list of MOIS for many common cell lines. If you are unable to locate the known MOI for your cell line, GeneCopoeia recommends determining the MOI by first transducing your cells with lentivirus expressing a fluorescent reporter such as GFP or mCherry.

Cell line	Tissue	Cancer/cell type	Species	MOI
A431	Epithelial	Carcinoma	Human	5
A549	Lung	Carcinoma	Human	5
Astrocytes	Nervous system	Primary	Human	1
B16-F10	Epithelial	Melanoma, metastatic	Mouse	5
BMM	Bone Marrow	Primary	Human	8
BxPC-3	Pancreas, epithelial	Adenocarcinoma	Human	10
H3255	Lung	Carcinoma, NSCLC	Human	10
HCT116	Colon	Carcinoma	Human	5
HeLa	Cervix	Carcinoma, epitheloid	Human	3
HEK293T	Kidney	Tumor	Human	5
Hepa1-6	Liver	Carcinoma	Mouse	3
HMVEC	Endothelial	Endothelial, microvascular	Human	100
HT-29	Colon	Adenocarcinoma	Human	3
HUVEC	Umbilicus	Endothelial cells	Human	100
Jurkat	Blood	Leukemia, Acute T Cell	Human	10
LLC-1	Lung	Carcinoma	Mouse	6
LNCaP	Prostate	Carcinoma	Human	5
MM200	Skin	Melanoma	Human	5
MCF-7	Breast	Adenocarcinoma	Human	2

MDA-MB-231	Breast	Adenocarcinoma	Human	1
MM-AN	Skin	Melanoma, metastatic	Human	16
MMC	Breast	Carcinoma	Mouse	4
MRC-5	Lung, embryonic	Fibroblasts	Human	1
NB4	Blood	Leukemia, acute promyelocytic	Human	10
PC12	Adrenal gland	Pheochromocytoma	Rat	20
SKOV-3	Ovary	Adenocarcinoma	Human	15
U-2 OS	Bone	Osteosarcoma	Human	5

Table 1. Lentiviral particle MOIs for some commonly used cell lines.

Transduction of Target Cells with Lentivirus

The transduction efficiency depends upon the target cells and experimental procedure.

Day 1: Plate cells

1. Plate 2–10 x 10⁴ of the target cells per well in a 24-well plate 24 hours prior to viral infection. Use 0.5 ml of cell specific medium supplemented with 5% heat-inactivated fetal bovine serum, and penicillin-streptomycin (optional) for each well. Incubate the cells at 37°C with 5% CO₂ overnight.

Note:

Make sure the cells reach 70-80% confluence at the time of transduction. The actual cell number to be plated depends on the cell type.

Day 2: Transduce target cells

2. For each well, prepare 0.5 ml of virus suspension diluted in complete medium with Polybrene (Millipore Sigma) at a final concentration of 5–8 µg/ml.

Note:

Use several dilutions of pseudoviral stock (0.1 µl to 100 µl). We recommend gradient dilutions of 0.1 µl, 0.3 µl, 0.5 µl, 0.7 µl, 0.9 µl for purified particles. In addition, we recommend including a transduction with the eGFP control and other appropriate positive and negative controls. Mix the virus with the medium gently by inverting the tubes several times. Do not vortex.

3. Infect the target cells by removing the old culture medium and replacing it with 0.5 ml of diluted viral supernatant. For one well (mock well control), add 0.5 ml of complete medium with Polybrene. Place the plates in a 37°C incubator with 5% CO₂ and incubate cells overnight. (Optional: Place the plates for 2 hours at 4-8°C; then transfer the plates to a 37°C incubator with 5% CO₂ and incubate cells overnight.)

Note:

Incubating cells with lentivirus for 2 hours at low temperatures can significantly increase the transduction efficiency. However, this step may be omitted if the cells cannot tolerate low temperatures.

Day 3: Replace medium/Split cell culture

4a. Replace the old medium with 0.5 ml of fresh complete medium (without Polybrene). Continue to 5a.

4b. Alternatively, split the cells 1:5 to 1:25 depending on the cell type by trypsinizing and re-seeding the cells onto 6-well plates or 10-cm culture dishes. Continue incubating for 48 hours in cell-specific medium. Continue to 5b.

Day 5: Analyze transduced cells or start drug selection of stably transduced cells

5a. The infected target cells can be analyzed for transient expression of transgenes using an appropriate biological assay. If you have used an internal eGFP control, determine the percentage of infected cells by counting fluorescing cells by flow cytometry or with a fluorescent microscope.

5b. To select stably transduced cells, replace old medium with fresh complete medium containing the appropriate selection drug every 3–4 days until drug-resistant colonies become visible (generally 7–14 days after selection).

Single clone isolation

Whether you are transfecting the cells or using lentivirus, GeneCopoeia recommends isolating stable clones grown from single cells. The reason is that if you want to obtain a cell line with your gene product completely eliminated, single clone isolation will rid the population of cells in which the gene is either incompletely knocked out or that went untransfected, and that carry unwanted background mutations. Clonal isolation is followed by an expansion period to establish a new clonal cell line. Note that cell types can vary substantially in their responses to single-cell isolation, so literature specific to the cell type of interest should be consulted.

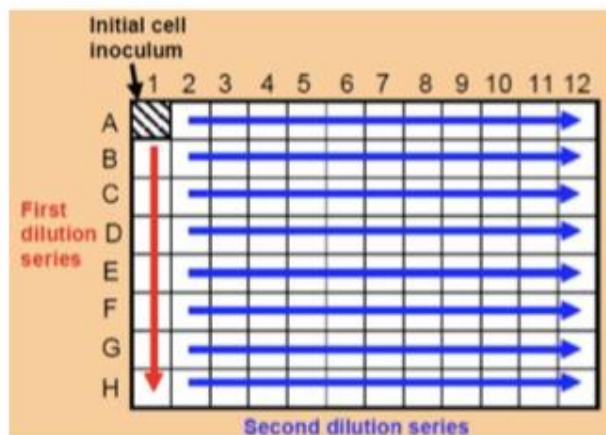
Single clone isolation method A: Cloning cylinders (recommended for adherent cells)

1. 24-48 hours after transfection, trypsinize the cells and transfer to a sterile 15 mL centrifuge tube.
2. Centrifuge the cells at 1,000 x g for 3 minutes.
3. Resuspend cell pellet in 5 mL complete growth medium.
4. Count the number of live cells in a hemocytometer to determine the cell density. Use trypan blue to exclude dead cells, which take up the dye and turn blue.
5. Dilute cells in 40 mL of complete growth medium to a density of ~1-2 cells/mL in a sterile 50 mL centrifuge tube. Make 10-fold serial dilutions from the starting suspension in order to achieve this cell density.

6. Dispense 10 mL of diluted cell suspension into each of 4 10 cm tissue culture dishes. If your transfection efficiency is low and you did not enrich for transfected cells, you will need to scale this up accordingly in order to obtain and screen more clones.
7. Incubate the plates at 37°C until cells have grown into colonies that are large enough to remove using sterile cloning cylinders. This can take several (3-6) weeks, depending on the growth rate of the parent cells.
8. Remove single clones from the plates using sterile cloning cylinders. Transfer each clones to 1 well of a 24-well plate containing 0.5 mL of complete growth medium.

Single clone isolation method B: Serial dilutions (for both adherent and suspension cells). Please note that serial dilution provides no guarantee that the colonies arose from single cells. A second round is advised to increase the likelihood that clonal isolation is successful.

1. Fill each well of a sterile 96-well plate with 100 μ L of medium except for well A1, which should remain empty.



2. Add 200 μ L cell suspension to well A1. Mix 100 μ L from A1 with the medium in well B1. Avoid bubbles. Continue this 1:2 dilution through column 1. Add 100 μ L of medium back to column 1 so that wells A1 through H1 contain 200 μ L.
3. Mix cells and transfer 100 μ L of cells from column 1 into column 2. Mix by gently pipetting. Avoid bubbles. Repeat these 1:2 dilutions through the entire plate. Bring the final volume to 200 μ L by adding 100 μ L of medium to all but the last column of wells.
4. Incubate plates undisturbed at 37°C.
5. Cells will be observable via microscopy over 3 days and be ready to score in 5-8 days, depending on the growth rate of cells. Mark each well on the cover of the plate indicating which well contains a single colony. These colonies can later be subcultured from the well into larger vessels.

Tech Notes:

- 1) Adding 4000 cells in well A1 (2×10⁴ cells/mL) is a good starting concentration. Increase the concentration for more difficult to grow cell lines.
- 2) If the reporter gene is fluorescent, determine which of these colonies express it. If the reporter gene is not observable you will have to wait until later in the culture process.
- 3) Label each well with a single colony using a unique identification number and record this number on the plate and in your notebook.

Clone screening

GeneCopoeia recommends using the IndelCheck™ Insertion or Deletion Detection system for clone screening, followed by DNA sequencing of the mutant allele(s).

1. Grow candidate clones to approximately 95% confluency (for adherent cells) or to a density of ~1 x 10⁷ cells /mL (for suspension cells).
2. Split cells to new plates or flasks, 2 for each candidate clone.
3. Harvest cells for each clone and use the IndelCheck™ kit to screen for clones that contain an indel. Follow the IndelCheck™ kit instructions for DNA isolation or cell lysis, PCR amplification, and T7 Endonuclease I digestion. Do not use all of the PCR product DNA for the IndelCheck assay. Save some for DNA sequencing of positive clones. Use the same PCR primers flanking the sgRNA target site(s) that were used for sgRNA validation.
4. Subclone the untreated PCR products into *E. coli* and sequence the insert DNA. Sequence 4-8 *E. coli* subclones.
5. Analyze the sequence of each *E. coli* subclone. A successful CRISPR-mediated modification will result in an insertion or deletion of 1 or more base pairs.
6. If a complete, multi-allelic knockout is desired, then the following criteria must be met by the results of the DNA sequencing:
 - a. All *E. coli* subclones must carry an indel mutation. No wild type alleles can be present. Note that, if bi- or multi-allelic modification has occurred, then it is likely that each copy of the gene will have a distinct allele, due to the random nature of NHEJ. In addition, if your cell line has more than two copies of your target gene, then you should observe the presence of the same number of distinct indel alleles. If you do not observe the presence of the same number of distinct indel alleles, then we recommend isolating and sequencing more *E. coli* subclones. An exception occurs for genes that are essential for cell viability, in which case it will be unlikely to isolate cell line clones that lack a wild type allele.
 - b. All indel mutations must cause a frameshift. While it is possible that in-frame indels can lead to gene knockout, they can also cause expression of an aberrant protein, which in turn could cause an undesirable phenotype. It is far more likely that a frameshift allele near the initiator ATG will cause complete gene knockout, since either a severely truncated protein will be produced and degraded, or that the mRNA will be eliminated through nonsense-mediated decay.