

The CRISPR-Cas technique has been used to generate gene knock-out in many mammalian cell lines. Here, we showed detail protocols for generation of gene knock-out in HEK-293T cells by transient transfection method and lentiviral transduction method. The conditions may vary for different cell line(s).

1. Preparation of cells (for both plasmid transfection/virus transduction). Plate HEK-293T cells onto 24-well plates in DMEM 16–24 hrs before transfection. Seed the cells at a density of 1×10^5 cells per well in a total volume of 1 ml. (Note: scale up or down according to different cell line as needed. Do not plate more cells than the recommended density, as doing so may reduce transfection/transduction efficiency.)

2-1. Plasmid transfection method. On the day of transfection, cells are optimal at 60–80% confluency. Cells should be transfected with optimal transfection reagent (e.g., Lipofectamine 2000 or TransIT-LT1 for HEK-293T cells) according to the manufacturers' instructions and the transfection efficiency varies according to cell type and the transfection reagent used. For cells with low transfection efficiency, please use electroporation or virus transduction method for plasmids delivery. Prepare 500 ng of plasmid for the expression of sgRNA(s) and Cas9 protein for transfection (and 500 ng of surrogate reporter plasmid if you are planning to use cell sorter to enrich the cell population with gene editing). If you are transfecting more than one plasmid (for example, two vector system for sgRNA and Cas9 expression), mix them at equimolar ratio and use no more than 1 μ g of total DNA. Control sgRNA might be used in the negative control experiment if needed.

2-2. Lentiviral transduction method. In the case of lentiviral transduction method, cells are optimal at 60–80% confluency on the day of transfection. For adherent cells, cells should be spin-infected with lentivirus(es) expressing sgRNA and Cas9 in culture medium containing polybrene. The concentration of polybrene would vary according to cell type and the regular concentration used in HEK-293T cells and A549 cells is 8 μ g/ml. For suspension cells, add lentivirus(es) into the culture medium and both spin-infection and polybrene are not necessary for lentiviral transduction. A lentivirus expressing control sgRNA might be used in the negative control experiment if needed.

3-1. Antibiotic selection (for both plasmid transfection/virus transduction). At 16–24 hrs post-transfection/post-transduction, the culture medium should be replaced with fresh medium containing puromycin to remove non-transfected/non-infected cells. The working concentration of puromycin varies according to

different cell line used, e.g., the optimal concentration of puromycin for the treatment of HEK-293T cells is 2–2.5 µg/ml. Incubate the cells for a total of 48–72 hrs post-transfection/post-transduction before passaging them for downstream applications. After 3–7 days of puromycin selection, single cell clones are isolated by limiting dilution into 96-well plates.

3-2. Enrichment population by cell sorter (for plasmid transfection only). There is an alternative way to enrich the population of cells with gene editing by cell sorter. A surrogate reporter has been demonstrated to be an effective and reliable tool enabling one to selectively enrich genetically modified cells. In brief, at 72 hrs post-transfection, the cells are harvested for cell sorting by indicated fluorescence, e.g., mCherry fluorescence in our assay system. Antibiotic selection is not necessary. For those cells with CRISPR/Cas9-mediated gene editing, the mCherry fluorescent gene in the surrogate reporter may become in-frame and then expressed. The mCherry is a monomeric fluorescent protein with peak absorption/emission at 587 nm and 610 nm, respectively. Cells with mCherry fluorescence will be collected by cell sorter to enrich the population of cells with possible editing in the endogenous gene. After 3 days of recovery from sorting, single cell clones are isolated by limiting dilution into 96-well plates.

4. Limiting dilution (for both plasmid transfection/virus transduction). The cell line should tolerate being single cell diluted when plating 96-well plates at 1 cell per well in standard cell culture media. To make sure single cell clones will be picked up by this method, prepare cells by well trypsinization and resuspend cells into DMEM in 50 ml conical bottom tubes with the cell density of 1 cell per ml. Mix well then transfer diluted cells by dispensing 100 µl/well in all 12 columns of five to ten 96-well plates using a multichannel pipette. Transfer 96-well plates to a humidified incubator at 37°C with 5% CO₂. Add 100 µl of fresh culture medium every week and visually inspect the 96-well plates periodically from 7 to 14 days under a microscope to assess if colonies are establishing. Note that only 5–10 colonies will be established in one 96-well plate. Expand and further culture those cell colonies in 24-well or 12-well plates until the cell numbers are enough for genomic DNA isolation (for genomic DNA PCR/sequencing) and protein extraction (for western blot analysis). The remaining pooled cells could be used for T7E1 assay (for validation of NHEJ efficiency) or western blot analysis (for gene knock-out analysis in pooled cells).

5. T7 Endonuclease I assay (for both plasmid transfection/virus transduction). Pooled cells are harvested to isolate genomic DNA for T7E1 assay. Prepare

genomic DNA and setup a 50 μ l PCR reaction by using 100 ng genomic DNA and 0.3 mM proper genomic PCR primers spanning the Cas9 cleavage site(s). The DreamTaq Green from Thermo Scientific (#K1081) is recommended. After 40 cycles of PCR reaction, perform additional denaturation and reannealing in a PCR machine to obtain the denatured/reannealed PCR products. Transfer 20 μ l denatured/reannealed PCR products to new PCR tubes and add 0.3 μ l of T7E1 (NEB#M0302L) into each sample. Digest the denatured/reannealed PCR products at 37°C for 40 min. (Note: Longer incubation time will result in non-specific digestion.) The NHEJ efficacy could be analyzed by electrophoresis in a 2% agarose gel.

6-1. Validation gene knock-out by western blot analysis (for both plasmid transfection/virus transduction). If your antibody is good enough (with high specificity), we highly recommend using western blot analysis to validate gene knock-out phenotype among those single cell clones. Cell lysates prepared from either single cell clones or pooled cells are separated on SDS-PAGE gels, transferred onto nitrocellulose paper and incubated with primary antibodies. The missing of specific band in the western blot indicates that the target gene has been successfully knocked-out in the single cell clone. Two or more single cell clones with gene knock-out phenotype should be picked-up and presented in one set of experiment.

6-2. Validation gene knock-out by genomic DNA PCR/sequencing method (for both plasmid transfection/virus transduction). Prepare genomic DNA and setup a 20 μ l PCR reaction by using 100 ng genomic DNA and 0.3 mM proper genomic PCR primers spanning the Cas9 cleavage site(s). The DreamTaq Green from Thermo Scientific (#K1081) is recommended. After 40 cycles of PCR reaction, check PCR specificity by taking 5 μ l of PCR product for electrophoresis in a 1% agarose gel. After PCR clean-up, PCR product is ready for sequencing validation by using proper sequencing primer. For PCR products from heterozygous genomic DNA (heterozygous editing outcomes in different alleles), the PCR products should be sub-cloned into a plasmid cloning vector for further sequencing validation.