GeneCopoeia™
Expressway to Discovery

Protocol ● CRISPR Genome Editing In Cell Lines

Protocol 2: HDR donor plasmid applications (gene knockout, gene mutagenesis, gene tagging, Safe Harbor ORF knock-in)

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) http://www.genecopoeia.com/product/indelcheck-detection-system/), 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. 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.
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 sgRNA-containing 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. Use selection only for the donor 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.

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 (total of all plasmids being co-transfected) 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 CO_2 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.

Single clone isolation

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 modified, single clone isolation will rid the population of cells in which the gene is either incompletely modified 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).
Note: All media used at this stage must contain antibiotic that selects for the donor plasmid.

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 containing antibiotic that selects for the donor plasmid in a sterile 50 mL centrifuge tube.
6. Make 4 10-fold serial dilutions from the starting.
7. Dispense 10 mL of each 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.

8. Incubate the plates at 37^{0}C until cells have grown into colonies that are large enough to remove using sterile cloning cylinders, from plates carrying 10-20 colonies each. This can take several (3-6) weeks, depending on the growth rate of the parent cells.
9. Remove 20 single clones from the plates using sterile cloning cylinders. Transfer each clone 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. Note: All media used at this stage must contain antibiotic that selects for the donor plasmid.

1. Fill each well of a 4 sterile 96-well plates with 100 µL of medium, except for well A1, which should remain empty.

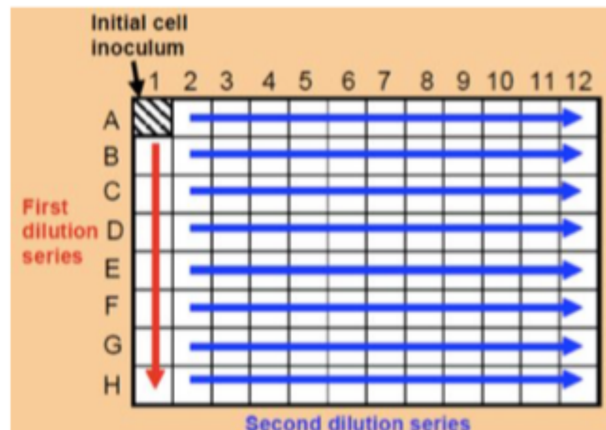

Figure 1. Serial dilution method for isolating singe clones.

2. Add 200 µL cell suspension to well A1. Mix 100 µL from A1 with the medium in well B1. Avoid bubbles. Continue this 1:2 dilution through column 1. Add 100 µL of medium back to column 1 so that wells A1 through H1 contain 200 µL.

3. Mix cells and transfer 100 µL 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 µL by adding 100 µL 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. Design PCR primers that flank both donor:chromosome junctions (Figure 2). At the 5' or "left" junction, the forward primer must recognize sequence from the chromosome outside of the region of homology with the plasmid. The reverse primer target sequence will only be found in the plasmid (for example, from the drug resistance gene). Conversely, at the 3' or "right" hand junction, the forward primer target sequence will only be found in the plasmid (for example, from the drug resistance gene), while the reverse primer must recognize sequence from the chromosome outside of the region of homology with the plasmid.

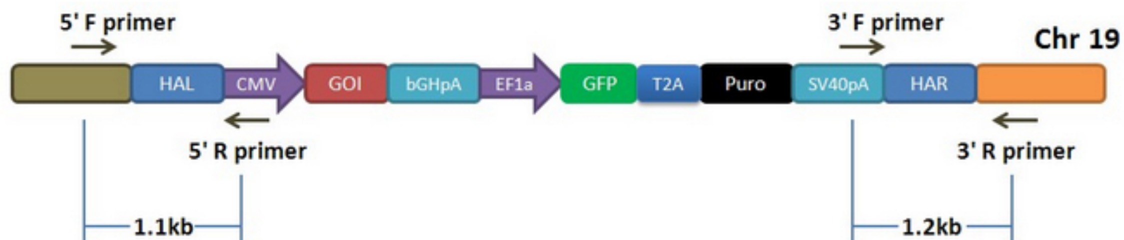

Figure 2. Junction PCR primer design strategy.

2. Grow candidate clones to approximately 95% confluency (for adherent cells) or to a density of ~1 x 10^7 cells /mL (for suspension cells).
3. Split cells to new plates or flasks, 2 for each candidate clone.
4. 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, and PCR amplification. Successful amplification using this strategy will occur only if the donor plasmid integrated at the correct site (Figure 3).

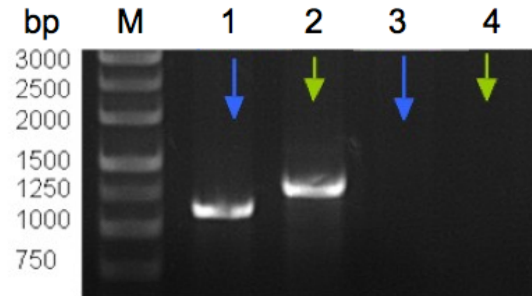

Figure 3. Junction PCR results. Lanes 1 and 2 are 5' and 3', respectively. Junction PCR products from a single puromycin resistant clone. Lanes 3 and 4 are 5' and 3', respectively. Junction PCR products from a second single puromycin resistant clone. Positive PCR reactions in lanes 1 and 2 indicate that donor plasmid integration occurred in the correct locus in that particular clone, whereas the negative PCR reactions in lanes 3 and 4 indicate that puromycin resistance arose in that clone from random integration of the donor plasmid.

5. Do not use all of the PCR product DNA for the junction PCR assay. Save some for DNA sequencing of positive clones.
6. Sublcone the untreated PCR products into *E. coli* and sequence the insert DNA. Sequence 3-4 *E. coli* subclones for each junction.
7. Analyze the sequence of each *E. coli* sublcone to ensure that the junction sequences are correct.
8. Do a second PCR experiment to confirm that the sequence being knocked in is present, using appropriate PCR primers.
9. If a complete, multi-allelic knockout is desired, then all *E. coli* subclones must carry a modification. No wild type alleles can be present. Note that, if bi- or multi-allelic modification has occurred, then it is possible that one or more alleles will contain the modification you are knocking in, while one or more of the remaining alleles will contain an indel (insertion or deletion) resulting from CRISPR-mediated nonhomologous end joining (NHEJ). 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 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 may be unlikely to isolate cell line clones that lack a wild type allele.
10. Rule out random integration of the donor plasmid. Even if you isolate cell line clones carrying the insertion at the correct chromosome site, it is possible that clones sustained a second, random integration, which is often undesirable. In such cases, the entire plasmid will integrate into the chromosome. To detect such random integrations, design one or more pairs of PCR primers that recognize the plasmid backbone only. Positive results from such PCR reactions indicate that the cell line clone sustained one or more random integrations of the donor plasmid.

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