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Protocols for CRISPR plasmid (dRGENs)

1. Preparation of CRISPR plasmids.

- Expression vectors for guide RNA (pRGEN-sgRNA-U6) and Cas9 gene (pRGEN-Cas9-CMV) are ampicillin-resistance and stable in general E.coli strains such as DH5a or XL1.
- The yield of pRGEN-sgRNA-U6 plasmids purification could be lower than usual practice. Use less volume of suspension/elution buffers.

2. Target PCR condition setup.

- Detailed procedure of PCR condition setup and mutation detection assay can be found in the T7E1 assay protocol (separate file)

3. Validation of CRISPR plasmid activity.

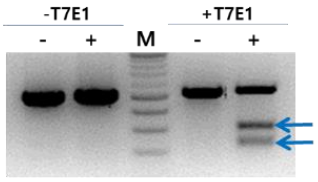
- The purpose of this step is to confirm and quantify the activity of CRISPR plasmid in cell. It also helps to have this step to practice and setup the T7E1 assay and other mutation analysis assay which are broadly used in the process of gene modification in cell lines.
- Common cell lines that have well-established DNA delivery protocol (such as 293T and HeLa) are good starting to validate the activity of CRISPR plasmid.

1. Day 1. Transfect CRISPR plasmid into target cell.
   : The ratio of CRISPR plasmids and Cas9 expression plasmids can be 1:1 ~5:1.
2. Day 3~4. Prepare genomic DNA from transfected cells.
3. Analyze the mutation at target locus by a T7E1 assay.

Expected results

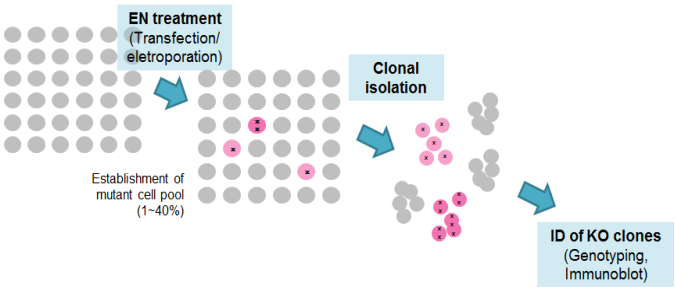
MED12 CRISPR plasmid

PCR product	507 bp
Expected cut	287 bp
	220 bp



- Transfection of total 1ug DNA (CRISPR plasmids 500ng, Cas9 expression plasmid 500ng) into HEK293T cells in 24 well using Lipofectamine 2000 (reverse transfection)
- Genomic DNA prep at 48h post-transfection.
- Mismatch sensitive nuclease assay (T7E1 assay)

4. Gene Knockout Cell Establishment.



1) Transfer CRISPR plasmids to Target Cells.

- Any DNA delivery method (transfection, electroporation) optimal for your cell line can be used for the delivery of CRISPR plasmid.
- The recommended ratio of guide RNA expression plasmids and Cas9 gene expression plasmids is 1:1 ~ 5:1.

2) 2~3 days after CRISPR plasmid treatment, plate appropriate density of cells to isolate monoclonal cell colonies.

- The colony formation efficiency could vary among cell lines. Thus, optimal density of cell population needs to be determined empirically.

Dish method: plate 50, 250, 1000, and 5000 cells / 10 cm dish (2 plate / cell population)

Limiting dilution method: plate 0.4 cell / well of 96 well plate (2~3 plate)

(It is recommended to proceed to TEST according to each cell. And this process is mean screening after colony seeding.)

3) 10~20 days after plating, isolate and expand mono-clonal cell colonies (50~100 colonies are recommended).

4) Prepare genomic DNA from each clone at 48 well plate ~ 12 well plate.

5) Identify the knockout cell clones by genotyping and immunoblot analysis.

- Most mutation induced by CRISPR plasmid at target site is small deletions and insertions (-20 bp~+10 bp). When these mutations are causing the frameshift, it will function as knockout mutation
- Cell lines usually contains more than 2 alleles (polyploidy)
- The complete knockout cell lines will have frameshift mutation on all alleles of target gene.
- These knockout cell lines can be identified by analyzing the mutation (genotyping) or/and by analyzing target gene expression
- T7E1 assay screening (detailed protocol from separate file)
  : Screening of isolated colonies by T7E1 assay will identify cell clones with mutation at target site but will not be able to discern the heterozygous and homozygous knockout cells

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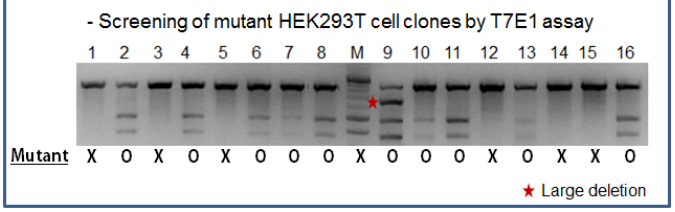
Expected results

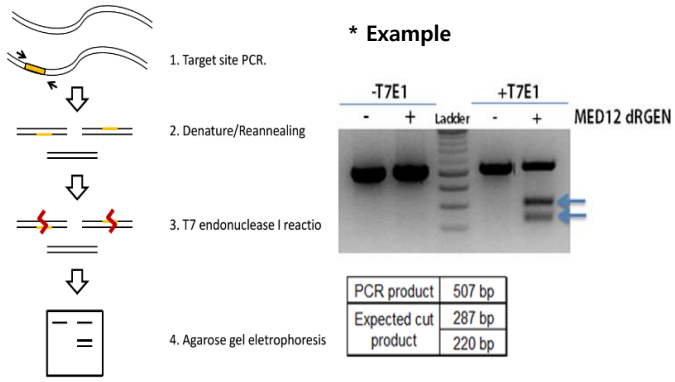


- TA-cloning and sequencing

: To confirm the frameshift mutation in all alleles (Homozygous knockout clones), amplified target locus are cloned into TA-vectors and multiple (usually >20) cloned alleles are sequenced and aligned.

Protocols for T7E1 assay

T7 endonuclease I is a surveyor nuclease. T7 endonuclease recognizes mismatched DNA, heteroduplex DNA. And T7 endonuclease I cleaves at the first, second or third phosphodiester bond that is 5′ to the mismatch.



I. Genomic DNA Extraction

II. PCR Amplification

1. PCR amplify the genomic DNA purified from transfected CRISPR plasmid & Cas9 plasmid.
2. Check PCR on 0.8% agarose gel.

DNA polymerase Taq	0.2 ul
10X buffer	2 ul
dNTP (10mM)	0.4 ul
Primer-F (10pmol)	0.5 ul
Primer-R (10pmol)	0.5 ul
gDNA	50~100 ng
DW	Up to 20 ul

Initial Denaturation	95℃	4:00	
Denaturation	95℃	0:30	35~40 Cycles
Annealing	X℃	1:00	
Extension	72℃	1:00	
Final Extension	72℃	10:00	
Hold	4℃	∞	

III. T7E1 Assay

1. Denature and annealing PCR reaction.
2. Take 15 μl PCR reaction and add 0.2~0.8 μl T7E1 enzyme to each reaction. Incubate at 37℃ 15 minutes.
3. Run the digestions on 2% agarose gel.
4. Visualize with UV illuminator.

Denature and annealing condition	
95℃	4:00
95℃ to 85℃	-2℃/s
85℃ to 25℃	-0.1℃/s

PCR reaction	15 ul
T7E1 enzyme (ToolGen)	0.2~0.8 ul
10X T7E1 buffer	2 ul
DW	Up to 20 ul