Protocols for CRISPR Plasmid

1. Validation of CRISPR plasmid activity.

Transfection of total 1ug CRISPR plasmid into HEK293T cell in 24 well using Lipofectamine 2000 (transfection or revers transfection).
Genomic DNA prep at 48h post transfection.
Mismatch sensitive nuclease assay (T7E1 assay)

2. T7E1 assay

T7 endonuclease I is a surveyor nuclease. T7 endonuclease recognizes mismatched DNA, heteroduplex DNA.

1) Genomic DNA Extraction

2) PCR Amplification

A. PCR amplify the genomic DNA purified from transfected CRISPR plasmid.

B. Check PCR on 1.5% (or 1%) agarose gel.

Step	Temperature	Time
Initial Denaturation	95℃	5 min
35 cycles	95℃	30 sec
	X ℃	30 sec
	72℃	30 sec
Final Extension	72℃	10 min
Hold	4℃	∞

3) T7E1 Assay

A. PCR amplification.

B. Hybridization of PCR products.

C. Take 10 μl PCR reaction and add 0.3~0.5 μl T7E1 enzyme to each reaction. Incubate at 37℃ 20 minutes.

D. Run the digestions on 2% agarose gel.

Hybridization condition		
Step	Temperature	Time
Denaturation	95℃	5 min
Annealing	95℃−85℃	-2℃/sec
	85℃−25℃	-0.1℃/sec
	72℃	30 sec
Hold	4℃	∞

3. 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.

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.
  (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. When these mutations are causing the frameshift, it will function as knockout mutation
- 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 genotyping or by analyzing target gene expression
- T7E1 assay screening.
  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.
- 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.