CRISPR Knockout / Knockin kit Validation
CRISPR needs Two Components: Cas9 and guide RNA

- Cas9, the nuclease
- Guide RNA (gRNA) ---
  20bp target specific scaffold---constant, can be built in a vector, gRNA scaffold

**Protospacer Adjacent Motif (NGG)**
All-in-one CRISPR/Cas9 vector

**pCas-Guide**

- Target sequence cloning
- Expresses Cas9

CAS9 + sequence specific gRNA targeted double-stranded break

Your Target Sequence

8.0 kb

pCas-Guide

Myc/DDK

Ori

U6 Promoter

AMPr

CMV Promoter
Genome Editing Is Achieved via Repair

CRISPR/Cas9

Unpredicted indels
- mutations
- Insertions/deletions
- Gene knockout

Desired
- Gene knock-out
- Specific mutations/SNP
- Deletion/insertion/tagging genes
- Knock-in (reporter gene)
- Promoter study

NHEJ

HDR

Donor template
CRISPR/Cas9 Tools

- CRISPR/Cas vectors
- Pre-designed donor vectors
- Genome-editing Knockout kit via CRISPR, genome-wide
  - 2 guide RNA vectors
  - 1 GFP-puro donor vector
    (gene specific homologous arms cloned)
  - 1 scramble control
KN210563 Was Used For Validation

ATG5 - human gene knockout kit via CRISPR

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Also for ATG5 (Locus ID 9474)

- cDNA Clone
- shRNA/siRNA
- Primer Pair
- Protein Request
- Antibody

Kit Components

- **KN210563G1**, ATG5 gRNA vector 1 in pCas-Guide vector, Target Sequence: AACTTGTTCACGCTATATC
- **KN210563G2**, ATG5 gRNA vector 2 in pCas-Guide vector, Target Sequence: AAGATGTGCTTCGAGATGTG
- **KN210563D**, donor vector containing Left and right homologous arms and GFP-Puro functional cassette. **Homologous arm and GFP-puro sequences**
- **GE100003**, scramble sequence in pCas-Guide vector
Diagram of CRISPR Knockout Kit

1. Target Sequence Cloned In pCas Guide Vector
   - Your Target Sequence
   - pCas-Guide 8.0 kb
   - U6 Promoter, miRNA, Bridge DNA Scaffold, CMV Promoter, Ori, Myc/DDK, CAS9

2. Donor Template DNA Containing Homologous Arms & Functional Cassette
   - eg. LHA, GFP, Loxp, Puro, Loxp, PGK, RHA, pUC

3. Genome Incorporation
   - LHA, Loxp, GFP, Puro, Loxp, RHA, PGK, Homologous Repair
   - Chromosome, ATG, Edited Chromosome

Cotransfection
Edited Chromosome –
gene knockout / GFP-Puro knockin

- Target gene is knocked out
- GFP under endogenous gene promoter
- Puromycin selection marker under PGK promoter
Protocols for targeted gene knockout using CRISPR Knockout / Knockin Kit

1. Cotransfection: one of the gRNA vector + donor vector
   Controls: 1). Scramble control + donor vector
             2). Donor only

2. Dilute cells containing donor vector ~ 20 days before puro selection
   Note: Since puro selection marker is under PGK promotion,
         Episomal and randomly integrated donor vector will also give puro resistance.
Diagram of diluting cells before puro selection

**P1**, 48 hr post transfection
- 1:10 split
- Grow for 3 days

**P2**, 5-day post transfection
- 1:10 split
- Grow for 3 days

**Optional**: Extract genomic DNA for PCR

**P3**, 8-day post transfection
- 1:10 split
- Grow for 3 days

**P4**, 11-day post transfection
- 1:10 split
- Grow for 3 days

**P5**, 14-day post transfection
- 1:10 split
- Grow for 3 days

**P6**, 17-day post transfection
- 1:10 split
- Grow for 3 days

**P7**, 20-day post transfection
- 1:10 split

Freeze or keep growing

If puro selection is needed again
Protocols for targeted gene knockout using CRISPR Knockout / Knockin Kit

1. **Cotransfection: gRNA vector + donor vector.**
   - Controls: 1). Scramble control + donor vector
   - 2). Donor only

2. Dilute cells containing episomal donor vector ~ 20 days post transfection

   Note: Since puro selection marker under PGK promotion, Episomal and randomly integrated donor vector will also give puro resistance.

3. Apply Puro selection. Isolate individual cell colonies
   - Note. Doses need to be determined by kill curve for each cell line
   - Donor vector alone can randomly integrate into the genome, but the efficiency should be much lower
Puromycin selection

After 5 splits, HEK293 cells were selected under 1 µg/mL puromycin for 5 days
4. Analyze puro positive cells.

A. WB to detect the knockout effect (better with single colonies)
B. Genomic PCR to verify GFP-puro integration, sequence the PCR products to confirm the integration.

Avoid Donor DNA contamination:
- F primer: upstream of the 5’ end of left arm
- Reverse primer: GFP region
Genomic PCR of GFP-puro Integration

Genomic DNA was extracted from cells 5 days post transfection before puro selection.
Sequencing Using The Forward Primer

Correct integration at 5’ end of left arm

WT Genomic sequence

Edited genome

Donor sequence
Correct Integration of GFP-puro Cassette

GFP replaced ATG5

WT Genomic sequence
Donor sequence
Edited genome

ATG5 ORF
GFP
Other Donor Vectors with different FP or Luciferase
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