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

Your Target Sequence

pCas-Guide
8.0 kb

pCas-Guide
• Target sequence cloning
• Expresses Cas9

Cas9 + sequence specific gRNA
targeted double-stranded break
Genome Editing Is Achieved via Repair

**CRISPR/Cas9**

- **Unpredicted indels**
  - mutations
  - Insertions/ deletions
  - Gene knockout

- **NHEJ**

- **HDR**

- **Donor template**

- **Desired**
  - Gene knock-out
  - Specific mutations/SNP
  - Deletion/insertion/tagging genes
  - Knock-in (reporter gene)
  - Promoter study
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
   - pCas-Guide
   - ATG
   - 8.0 kb

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

3. Genome Incorporation
   - Cotransfection
   - Chromosome
   - Edited Chromosome
   - ATG
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

Optional: Extract genomic DNA for PCR

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

- **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
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

Donor only

pCas-Scrambled +Donor

pCas-T1 +Donor

pCas-T2 +Donor

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
Correct Integration of GFP-puro Cassette

GFP replaced ATG5
Other Donor Vectors with different FP or Luciferase

1. Target Sequence Cloned In pCas Guide Vector
   - Your Target Sequence
   - pCas-Guide 8.0 kb
   - Cotransfection
   - Genome Incorporation

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

3. Genome Incorporation
   - LHA GFP Puro PGK RHA
   - Edited Chromosome
   - ATG

A. LHA GFP Puro PGK RHA
B. LHA RFP BSD' PGK Loxp RHA
C. LHA Luciferase Puro' PGK Loxp RHA
D. LHA BFP Neo' PGK RHA
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