KN2.0 CRISPR Knockout Kit Validation
All-in-one CRISPR/Cas9 vector

pCas-Guide

- Target sequence cloning
- Cas9 expression

Cas9 + sequence specific gRNA
targeted double-stranded break
KN2.0 CRISPR Knockout Kit

✓ 2\textsuperscript{nd} generation CRISPR knockout kit

✓ Higher knockout efficiency

✓ EF1a-GFP-P2A-Puro

✓ A specific kit for every gene locus
KN2.0 CRISPR Knockout Kit

Components:

- 2 gRNA vectors in pCas-Guide
- Linear donor DNA containing EF1a-GFP-P2A-Puro
Scheme of KN2.0 CRISPR Knockout Kit

- Target sequence
- Linear donor
- Cotransfection
- Chromosome
- Cas9/gRNA
- Donor can be inserted in forward or reverse direction
- Forward integration
- Reverse integration

Donor can be inserted in forward or reverse direction.
KN2.0 Edited Chromosome

- Donor inserted at the gRNA cutting site
- Endogenous gene knocked out
- GFP under EF1a
- Puro with P2A

Forward integration

Reverse integration
Human ATG5 Knockout using KN2.0 in HEK293

- gRNA sequence: AACTTGTTCACGCTATATC
- Linear donor:
1. Cotransfection: gRNA plasmid + donor

2. Dilute cells after transfection ~ 20 days before puro selection

Note: Since puro selection marker is under P2A and EF1α promoter, episomal and randomly integrated donor vector will also give puro resistance.
Diagram of Cell Passaging After Transfection

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

2. **P2**, 5-day post transfection
   - 1:10 split
   - Grow for 3 days
   - Optional: Extract genomic DNA for PCR

3. **P3**, 8-day post transfection

4. **P4**, 11-day post transfection

5. **P5**, 14-day post transfection
   - 1:10 split

Freeze or keep growing
If puro selection is needed again

**Puro selection**
Verification of Donor Integration by Genomic PCR

Forward integration

5F: AGTTGGACTGTGACAGGATTCACA
3F: CCTATGACCGAGTACAAGCCC
5R: CAGGTGGAAGTAATTCAAGGCAC
3R: CCAGAACGCATCATGACAACA

5' Junction (0.8 kb) 3' Junction (0.9 kb)

Reverse integration

5F: AGTTGGACTGTCAGGATTCACA
3F: CCTATGACCGAGTACAAGCCC
5R: CAGGTGGAAGTAATTCAAGGCAC
3R: CCAGAACGCATCATGACAACA

5' Junction (1.2 kb) 3' Junction (0.7 kb)

- Please note 5F and 3R are gene specific, and they should be designed based on the target genome sequence.

[Diagram showing forward and reverse integration sites with primer sequences and junction sizes]
Three days after transfection, un-transfected (WT) and transfected (G1 and donor) HEK293 cells were harvested and genomic DNA was extracted. For forward donor integration, PCR was performed using primer pairs 5F and 5R (5’ junction); 3F and 3R (3’ junction).
Verification of Donor Insertion in Reverse Direction

Three days after transfection, un-transfected (WT) and transfected (G1 and donor) HEK293 cells were harvested and genomic DNA was extracted. For donor reverse integration, PCR was performed using primer pairs 5F and 3F (5' junction); 5R and 3R (3' junction).
Single HEK293T cell clones were isolated after puromycin selection. Genomic DNA was extracted and PCR was performed using primer pair 5F and 3R. WT: untransfected cells. 1, 2, 3, 4, 5, 6: single colonies of G1 and donor transfected.

50% of Cell Clones Contain Donor Insertion

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<th>M</th>
<th>WT</th>
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- **With donor**
  - 3.4 kb (5F and 3R)
  - No donor or indels
- **No donor or indels**
  - 0.75 kb

Diagram:
- gRNA cutting
- GFP
- P2A
- Puro
- 5F
- 3R
- (0.75 kb)
0.75 kb PCR Fragments have Indels
0.75 kb PCR Fragments have Indels

2 insertions, one mutation

WT Genomic Sequence
GGACGAATTCCAACTTGTTTCACGCTAT

Guide sequence
AACTTGTTTCACGCTAT

Edited Genomic Sequence
GGACGAATTCCAACTTTTTCACGCTAT

Fragment base #110 is selected. Base 110 of 110
Human SHMT1 Knockout using KN2.0 in MIA PaCa-2

gRNA 1: TCAACATCACTGTCTTTGAG
gRNA 2: ACCACAGGTCAGCATCCTTG

Donor

CMV

Puro

This KN2.0 validation data are provided by a customer
MIA PaCa-2 cells were transfected with gRNA and donor DNA using lipofectamine 3000, selected with puromycin after passaging for 8 times. Genomic DNA was extracted from the stable pools and genomic PCR was performed using primers flanking the integration site (PCR fragment from endogenous locus will be 413 bp, with donor insertion will be 1.6 kb). Control DNA is from non-transfected cells.
Single cell clones were isolated from gRNA 1 and donor transfected MIA PaCa-2 stable pools. Genomic PCR was performed using primers flanking the integration site. Control DNA is genomic DNA from non-transfected cells.
The 0.4 kb Fragment Has Insertion of “A”

TGGAAGCTACACATGTTTTTCCCATTTTTTTAGGCAGCTTCGAACCAGTGCAATGACGATGCCAGTCAACGGGGCCC
ACAAGGATGCTGACCTGTGGTCCCTCACATGACAAGATGCTGGCACAACCC
CTCAAAGACAGTGATGTTGA
GGTGAGATTTTTTGGGGTCTTCACAGATTTTTTTATGTGGGACCTCATTATCCATTATAATTAGGG
ACAGCCTTGAATGTATTATCTCTGTGAGATTTAGGTGGCAGACAAAATGGCTACAAATCCTTTGAGGGTA
AAATTTAAAGATTTGCTGGGCTTCTTCAAGATGTGGGACACTGTGCAAGGTGCTCTGGAGAAAAGCGAGAAAGGG
GAGAGATCACTGACAGTTGACCCTTTCCTGACA

F-primer TGGAAGCTACACATGTTTTTCCCATTTTTTTAGGCAGCTTCGAACCAGTGCA
R-primer ACAAGGATGCTGACCTGTGGTCCCTCACATGACAAGATGCTGGCACAACCC
CTCAAAGACAGTGATGTTGA
GGTGAGATTTTTTGGGGTCTTCACAGATTTTTTTATGTGGGACCTCATTATCCATTATAATTAGGG
ACAGCCTTGAATGTATTATCTCTGTGAGATTTAGGTGGCAGACAAAATGGCTACAAATCCTTTGAGGGTA
AAATTTAAAGATTTGCTGGGCTTCTTCAAGATGTGGGACACTGTGCAAGGTGCTCTGGAGAAAAGCGAGAAAGGG
GAGAGATCACTGACAGTTGACCCTTTCCTGACA

hSHMT1 CRISPR V2_F: TGGAAGCTACACATGTTTTTCCC
hSHMT1 CRISPR V2_R: ACAGTTGACCCTTTCCTGACA
hSHMT1 gRNA1 RC: CTCAAAGACAGTGATGTTGA
hSHMT1 gRNA2 RC: CAAGGATGCTGACCTGTGGT

Legends:
1. hSHMT1 CRISPR V2_F
2. hSHMT1 CRISPR V2_R
3. hSHMT1 gRNA1 RC
4. hSHMT1 gRNA2 RC

The 0.4 kb Fragment Has Insertion of “A”
SHMT1 Biallelic Knockout was Confirmed on WB

Single clones, #6 and $11, and non-transfected MIA PaCa-2 cells (control) were subjected to WB with SHMT1 polyclonal antibody. NS- non-specific band. The same blot was blotted with β-actin antibody as sample loading control.