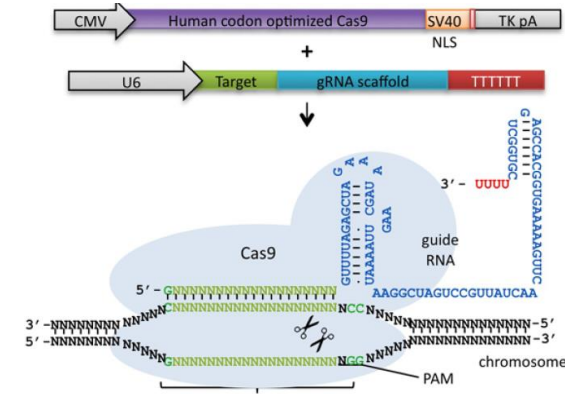


pCas-Guide System Validation in Genome Editing

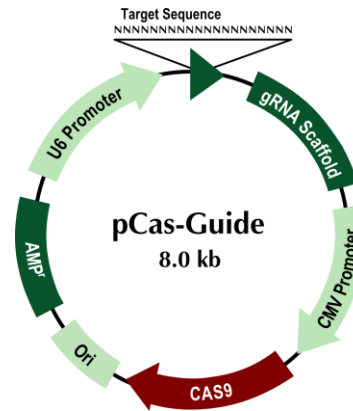
Tagging HSP60 with HA tag genome editing

The latest tool in genome editing – CRISPR/Cas9 – allows for specific genome disruption and replacement in a flexible and simple system resulting in high specificity and low cell toxicity. The system uses a nuclease, Cas9 in complex with guide RNA (gRNA) to cleave DNA in a sequence-specific manner upstream of the protospacer-adjacent-motif (PAM - the sequence NGG) in any genomic location.



RNA-Guided Human Genome Engineering via Cas9

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pCas9-Guide vector expressing human codon-optimized Cas9 and gRNA

The main purpose of this paper is to provide details how to tag a gene in the cellular genome using OriGene's pCas-Guide system.

I. The wild-type HSP60 C-terminal sequence and the desired C-terminal HA-tagged HSP60 sequence after genome editing

A. The wild-type HSP60 C-terminal sequence. Sequences in green are two targeting sequences for gRNA cloning; one before the stop codon (labeled with *) and one after the stop codon. Regarding how to design gRNA will be displayed below.

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TTA TTG GAT GCT GCT GGT GTG GCC TCT CTG TTA ACT ACA GCA GAA GTT GTA GTC ACA GAA ATT CCT 1787
L L D A A G V A S L L T T A E V V V T E I P 550
AAA GAA GAG AAG GAC CCT GGA ATG GGT GCA ATG GGT GGA ATG GGA GGT GGC ATG 1853
K E E K D P G M G A M G G M G G G M G G G M 572
TTC TAA CTCCTAGACTAGTGCCTTTACCTTTATTAATGAAGTGTGACAGGAAGCCCAAGGCAGTGTTCCTCACCATAACTTCAGA 1938
F * 573
GAAGTCAGTTGGAGAAAAATGAAGAAAAAGGCTGGCTGAAAATCACTATAACCATCAGTTACTGGTTTCAGTTGACAAAATATATAAT 2025
GGTTTACTGCTGTCATTGTCCATGCCTACAGATAATTTATTTTGTATTTTGAATAAAAAACATTTGTACATTCCCTGATACTGGGTA 2112
CAAGAGCCATGTACCAGTGTACTGCTTTCAACTTAAATCACTGAGGCATTTTTACTACTATTCTGTTAAAATCAGGATTTTAGTGCT 2199
    
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B. The desired C-terminal HA tagged HSP60 sequence after editing (sequence in red is the HA tag)

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TTA TTG GAT GCT GCT GGT GTG GCC TCT CTG TTA ACT ACA GCA GAA GTT GTA GTC ACA GAA ATT CCT 1787
L L D A A G V A S L L T T A E V V V T E I P 550
AAA GAA GAG AAG GAC CCT GGA ATG GGT GCA ATG GGT GGA ATG GGA GGT GGC ATG 1853
K E E K D P G M G A M G G M G G G M G G G M 572
TTC TAC CCA TAC GAT GTT CCA GAT TAC GCT TAA CTCCTAGACTAGTGCCTTTACCTTTATTAATGAAGTGTGACAGG 1929
F Y P Y D V P D Y A * 582
AAGCCCAAGGCAGTGTTCCTCACCATAACTTCAGAGAAGTCAAGTGGAGAAAATGAAGAAAAAGGCTGGCTGAAAATCACTATAAC 2016
CATCAGTTACTGGTTTCAGTTGACAAAATATATAATGGTTTACTGCTGTCATTGTCCATGCCTACAGATAATTTATTTTGTATTTT 2103
    
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GAATAAAAAACATTTGTACATTCTGATACTGGGTACAAGAGCCATGTACCAGTGTACTGCTTCAACTTAAATCACTGAGGCATTT 2190
TTACTACTATTCTGTTAAAAATCAGGATTTTAGTGCTTGCCACCACCAGATGAGAAGTTAAGCAGCCTTCTGTGGAGAGTGAGAATA 2277
ATTGTGTACAAAGTAGAGAAGTATCCAATTATGTGACAACCTTGTGTAATAAAAAATTTGTTTAAAGTTAAAAA 2364
AA 2366
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II. Designing pCas-Guide targeting sequences, gRNA

Since the insertion site is at the end of the C-terminus of HSP60, the double-stranded break site should be near the insertion site which is the stop codon “TAA” in the wild-type sequence.

Cas9-Guide system also recognizes the reverse complement sequence. For the targeting sequence in the reverse complement sequence, the cleavage site should be at the 5’ end of the corresponding forward targeting sequence.

To design two gRNA sequences, a designing tool at the Blue Heron website was used <https://www.blueheronbio.com/external/tools/gRNASrc.jsp>. A sequence of around 60 bp flanking the ‘TAA’ was copied and pasted to the sequence box of the designing tool. After clicking the Search button, the search results were shown below:

Location	Target Sequence	Pam	GC content
2-21(F)	5’ ATGGGAGGTGGTATGGGAGG 3’	TGG	61%
59-40(RC)	5’ TAAAGGTAAAGCACTAGTCT 3’	AGG	39%

F, forward sequence; RC, reverse complement

The two sequences were selected, and the corresponding oligo pairs (shown in green text in the WT HSP60 C-terminus sequence above) were ordered and cloned to the pCas-Guide vector to produce gRNA when transfected into cells. The constructs are named pCas-HSP60T1 and pCas-HSP60T2 respectively.

III. Designing the HSP60 editing donor DNA

Oligo DNA was used as the editing donor for homologous recombination to insert the HA tag at the C-terminus of HSP60 in the genome. The HA tag sequence is flanked by about 50 bp HSP60 sequence before and after the stop codon respectively (homologous arms for recombination).

Forward Oligo (sequence in red is the HA tag sequence):

5’ GAATGGGTGCAATGGGTGGAATGGGAGGTGGTATGGGAGGTGGCATGTTCTaccatac gatgttcagattacgct
TAACTCCTAGACTAGTGCTTTACCTTATTAATGAACTGTGACAGGAAGC 3’

Reverse complement Oligo (sequence in red is the HA tag sequence):

5’ GCTTCCTGTCACAGTTCATTAATAAAGGTAAAGCACTAGTCTAGGAGTTAagcgtaatctggaacatcgatgggta
GAACATGCCACCTCCCATACCACTCCATTCCACCCATTGCACCCATTC 3’

The two oligos were annealed to form double-stranded DNA following the protocol below:

In a PCR tube, add the following:

4 μ L Forward oligo (100 μ M stock)

4 μ L Reverse oligo (100 μ M stock)

4 μ L 10X annealing buffer

40 μ L dH₂O

Mix the solution and follow the steps to anneal the oligos:

94°C for 4 min

75°C for 5 min

65°C for 15 min

25°C for 20 min

After annealing, transfer the solution to a 1.5 mL tube and add 360 μ L of dH₂O.

Measure the DNA concentration using a Nano drop. The DNA is ready for use.

IV. Genome editing through transfection

1. Day 1, Seed cells
Approximately 18–24 hours before transfection, seed HEK293T cells in a 6-well plate in 2.5 mL complete growth media per well. Ideally cells should be 50% confluent prior to transfection.
2. Day 2, Transfection
 - a. In a 1.5 mL tube, add 0.75 μ g of pCas-Guide DNA and 0.75 μ g of the HSP60 HA donor DNA. Add 60 μ L of Opti-MEM to dilute the DNA by mixing completely.
 - b. Add 4.5 μ L of transfection reagent (Turbofectin 8.0) to the diluted DNA mixture. Pipette gently to mix completely and incubate at RT for 20 min
 - c. Transfer the Turbofectin 8.0/DNA mixture to a well in the 6-well plate prepared on day 1 drop-wise. Gently rock the plate back-and-forth and from side-to-side to achieve even distribution of the complexes.
3. Day 3
24hr post transfection, change the cell culture media with fresh media; grow cells at a CO₂ incubator for an additional two days.
4. Day 5
Split the cells to three identical 6-well plates. Grow the cells for three more days.
5. Day 8
The cells can now be analyzed for genome editing.

V. Analysis detecting the HA tag inserted in the cellular genome

Analysis I, Protein extraction and Western Blotting to measure HA-tagged HSP60 protein

1. Remove the media from each well, wash the cells with 2 mL PBS.
2. Add 100 μ L RIPA buffer and collect the cell lysates into a 1.5 mL tube.
3. Clear the cell debris by spinning down at 4000rpm for 4 min.
4. Take the supernatant, add 100 μ L of 2X protein loading buffer.
5. Run the lysates on a 4 -12% gradient SDS PAGE Gel.
6. Perform protein transfer onto a PVDF membrane using a standard protocol.

- Western blotting was performed using anti-HSP60 and anti-HA antibodies respectively.

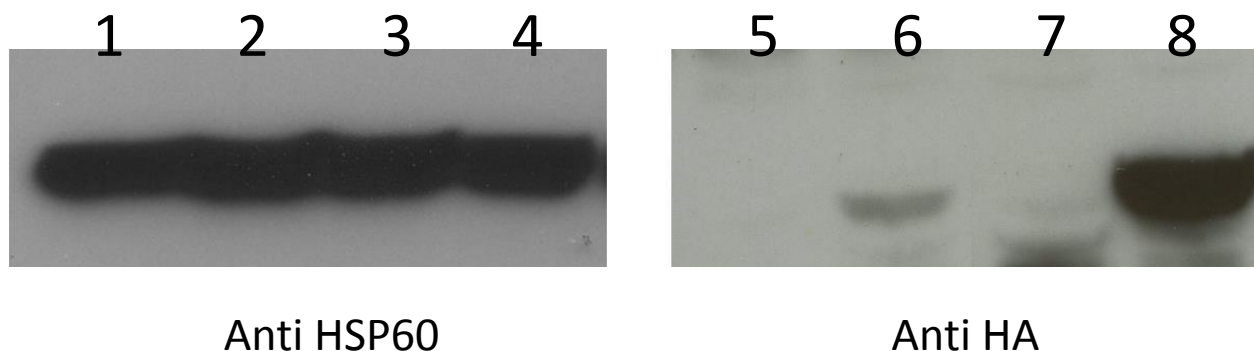


Fig. 1. Western blotting of pCas-Guide constructs and HSP60 donor DNA transfected HEK293 cells.

Lane 1 and lane 5: pCas-scramble plus HSP60 HA donor were transfected
 Lane 2 and lane 6: pCas-HSP60T1 plus HSP60 HA donor were transfected
 Lane 3 and lane 7: pCas-scramble plus HSP60 HA donor were transfected
 Lane 4 and lane 8: pCas-HSP60T2 plus HSP60 HA donor were transfected

Analysis II, Genomic DNA extraction and PCR to verify the HA tag sequence in the cellular genome

- Cells were harvested and genomic DNA was extracted using a genomic isolation kit.
- Measure the DNA concentration.
- Perform PCR using a pair of primers* detecting the HA sequence integration
- Run PCR products on a 1% Agarose Gel

*Note: to detect the targeted editing through PCR, a pair of PCR primers was designed. The forward primer is in the HA coding region and the reverse primer is in the HSP60 which is downstream of the donor sequence to avoid background contamination from the HSP60 donor oligo DNA. A positive PCR product with a correct size and subsequent sequencing of the PCR fragment will confirm the desired integration in the cellular genome.

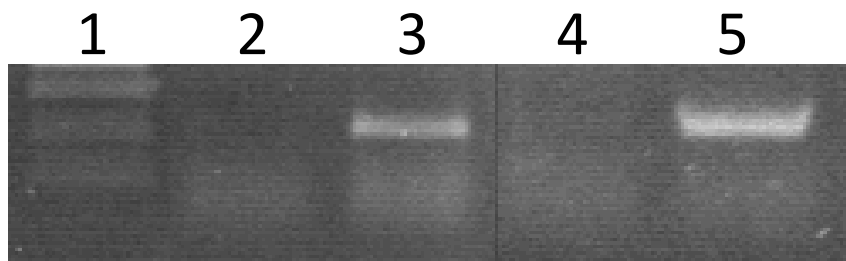


Fig. 2. PCR the genomic DNA from pCas-Guide constructs and HSP60 donor DNA transfected HEK293 cells.

Lane 1: DNA molecular Marker

Lane 2: pCas-scramble plus HSP60 HA donor

Lane3: pCas-HSPT1 plus HSP60 HA donor

Lane4: pCas-scramble plus HSP60 HA donor

Lane5: pCas-HSPT2 plus HSP60 HA donor

In summary, adding a tag to the endogenous gene is simple and affordable using pCas-Guide system. We used oligos as donor sequence in this tagging study. For other genome engineering, such as gene replacement with luciferase or GFP for promoter study and gene knock-in, rescue donor vectors can also be used. The rescue donor vectors can be ordered <http://www.blueheronbio.com/Services/Genome-Editing.aspx>.