CRISPR Stable Knock-in Cell Line Generation (Cat. No. C408)

Case Study: Using CRISPR to Knock-in Red Fluorescent Protein (RFP) gene into Human Embryonic Kidney Cells at the AAVS1 Safe Harbour Site

Summary

- An expression cassette containing RFP and puromycin resistance genes (pAAVS1-RFP-DNR) was knocked into the AAVS1 Safe-harbor site in HEK293 cells using CRISPR targeted genome editing via the HDR pathway. Gene insertion at a Safe-harbour site allows stable gene expression without any adverse effects on the fitness of the engineered cells.
- Genomic PCR confirmed Knocked–in RFP integration at AAVS1 Safe-harbor locus.
- RFP expression was confirmed in cells by fluorescence microscopy.

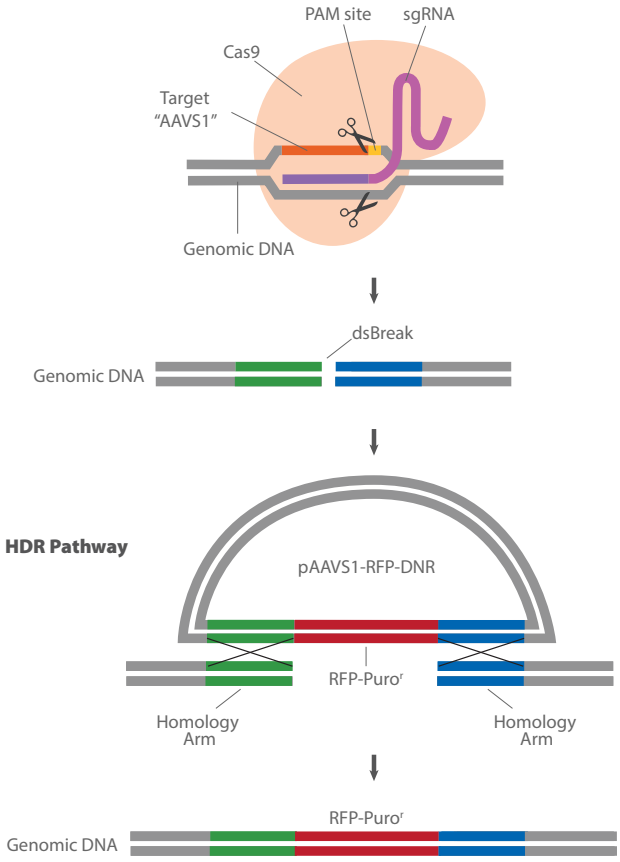

Figure 1 *CRISPR Knock-in requires expression of Cas9 and sgRNA to produce a double-stranded break. The repair template, shown here as pAAVS1-RFP-DNR, is used by the cell to repair the break using homologous recombination. The desired gene and selection marker (RFP and puromycin) included between the homology arms on the repair template will be integrated into the genome.*

Phase 1: Construction and Delivery of sgRNA, Cas9 and Repair Template

- An sgRNA was designed against the human AAVS1 Safe-harbor locus
- Software analysis was performed to ensure the sgRNA had no predicted off target binding sites. The selected sgRNA design, along with the CMV-promoter driven Cas9 gene, was cloned into pCas-Guide to make pCas-Guide-AAVS1 **(Figure 2)**.
- The pAAVS1-RFP-DNR donor plasmid was designed to contain the RFP-puromycin expression cassette, flanked on either side by homology arms of 600 bp **(Figure 2)**.
- HEK293 cells were co-transfected with both plasmids using DNAfectin transfection reagent.

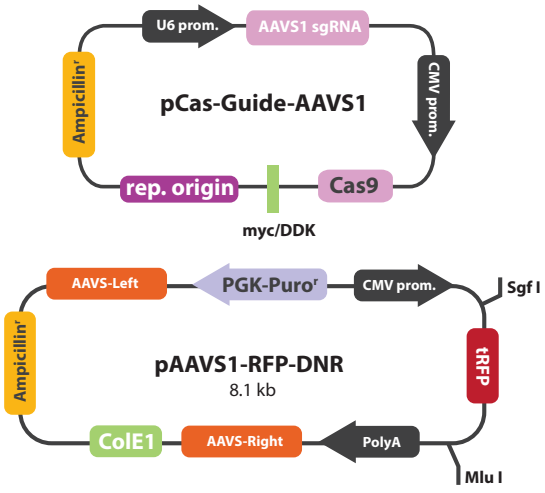

Figure 2 *Vector maps of pCas-Guide-AAVS1 and pAAVS1-RFP-DNR. pCas-Guide-AAVS1 is an all-in-one vector for co-expression of sgRNA and Cas9 in mammalian cells. Expression of sgRNA is driven by the U6 promoter, a strong constitutive Pol III promoter; while a CMV promoter drives the expression of the Cas9 enzyme. pAAVS1-RFP-DNR expresses puromycin resistance marker under the PGK promoter and RFP gene under the CMV promoter. The 5′ and 3′ AAVS1 homology arms ('AAVS-Right' and 'AAVS-Left') provide the cells with a template for Homology Directed Repair.*

Phase 2: Dilution of the Donor Plasmid and Resistance Marker Selection

- Transfected HEK293 cells were passaged ten times to dilute out the episomal donor vector.
- After these passages puromycin was added to the media to select for cells with successful knock-in of the RFP-puromycin resistance cassette.
- After 3-4 weeks of selection, >95% of HEK293 cells were expressing RFP.

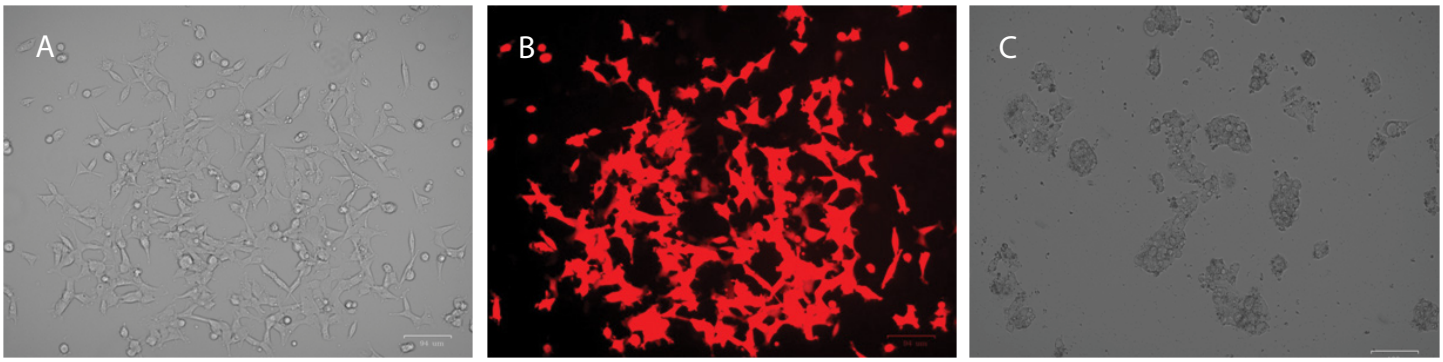

Figure 3 *After transfection, HEK293 cells were passaged ten times to dilute out the episomal vector, then grown in the presence of puromycin for 4 weeks. A) Cells transfected with both pCas-Guide-AAVS1 and pAAVS1-RFP-DNR were healthy after 4 weeks. B) Over 95% of these cells imaged in Figure 3 (A) expressed RFP. C) Control cells not transfected with the vectors died after puromycin treatment.*

Phase 3: Confirmation of Knock-in by Genomic PCR

- To confirm knock-in of RFP in the genomic DNA, a primer pair was designed with Primer 1 targeting the 5' homology arm upstream of RFP and Primer 2 targeting within the RFP-Puromycin resistance cassette.
- PCR product of 1.1 kb indicates successful knock-in at AAVS1 site; absence of PCR amplification indicates unsuccessful cassette insertion **(Figure 4)**.
- No PCR amplification was seen in the control cells ('WT cell') since Primer 2 could not anneal to the genomic DNA.

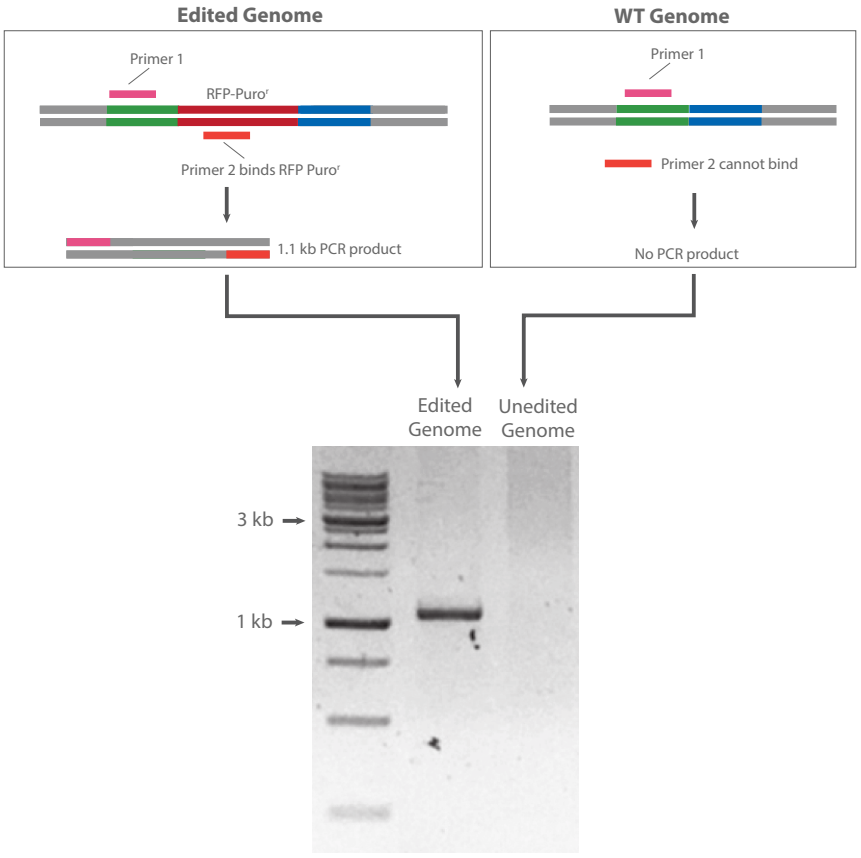

Figure 4 *Genomic PCR was used to confirm the knock-in of RFP. In edited cells, both primer 1 and primer 2 can bind, resulting in a 1.1 kb PCR product. No PCR product is formed in WT cells as primer 2 cannot anneal to the genomic DNA.*