

CRISPR Stable Knockout Cell Line Generation (Cat. No. C208)

Case Study: Using CRISPR to develop a biallelic LIF Knockout in Mouse Colon Carcinoma Cells

Summary

- LIF locus in a Mouse Colon Carcinoma Cell Line, was knocked out using CRISPR targeted genome editing.
- Surveyor assay and sequencing results showed genome editing.
- After monoclonal selection biallelic knockout was confirmed by sequencing.

Phase 1: Cas9 and sgRNA Delivery

- Three sgRNA were designed against mouse LIF locus (*Mus musculus*, NM_008501). Software analysis was performed to ensure the sgRNA had no predicted off targets binding sites. The selected sgRNA design was then cloned into the pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro All-in-One lentivector (**Figure 1**).
- Recombinant Lentiviruses were packaged using **abm's** second generation Lentiviral packaging system. A multiplicity of infection (MOI) of 5 was used to transduce the cells.

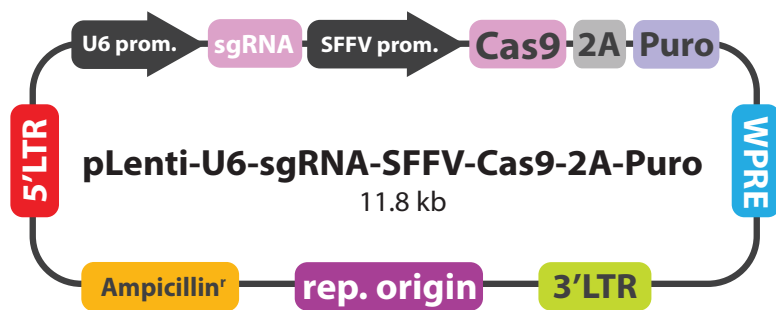
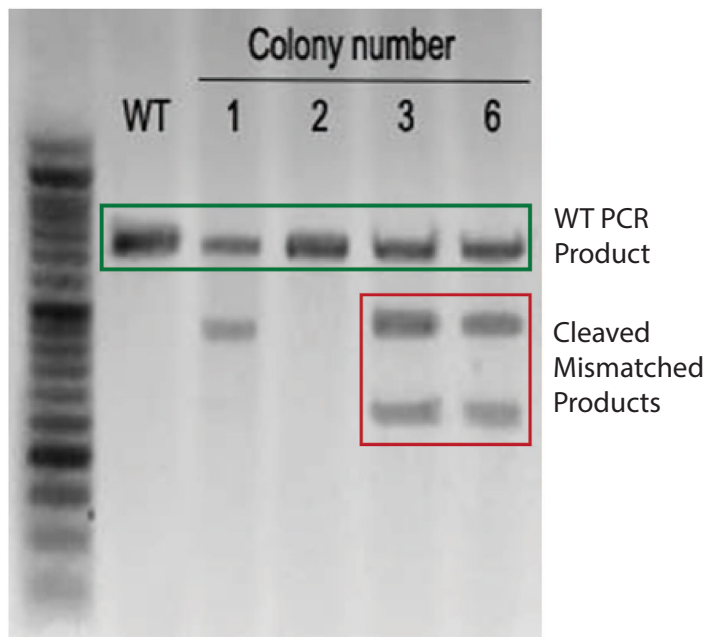


Figure 1 pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro lentivector is an all-in-one vector for co-expressing sgRNA and Cas9 in mammalian cells. Expression of sgRNA is driven by the U6 promoter, a strong constitutive Pol III promoter. An SFFV promoter drives expression of the Cas9-2A-Puro cassette. By using the Cas9-2A-Puro cassette, cells can be directly screened for expression of Cas9, as they will be resistant to Puromycin.

Phase 2: First Round of Colony Screening for Edited Clones

- Cell colonies are isolated after puromycin selection. Genomic DNA was extracted and the surveyor assay was performed to confirm genomic editing of the LIF locus.
- A single band in a surveyor assay at the wild-type (WT) size indicates no editing has occurred; two smaller bands (that sum to the length of the WT) indicate editing has taken place.
- The surveyor assay (**Figure 2**) indicated that Colony 3 and 6 were edited; colony 2 was not edited; and colony 1 was inconclusive.

Figure 2 The surveyor assay indicated that Colony 3 and 6 were edited; colony 2 was not edited; and colony 1 was inconclusive.



Phase 3: Sequence Analysis of the Edited Colonies

- PCR products from Colonies 3 and 6 were further analyzed via Sanger Sequencing to determine the nature of the knockout (**Figure 3**).
- For colony 3 only one mutant sequence was detected, indicating that these cells are likely only heterozygotic knockouts. In colony 6 two different mutant sequences were detected.

Sequence Alignment	490	500	510
WT	CGGCAACCTCATG	AACCAGATCAAGA	ATCAACTGG
sgRNA Sequence	-----CATG	AACCAGATCAAGA	-----
Colony 3	CGGCAACCTCATG	AACCAGATCAAGA	ATCAACTGG
Colony 6 Sequence 1	CGGCAACCTCATG	GGACCAGATCAAGA	ATCAACTGG
Colony 6 Sequence 2	CGGCAACCTCAT	-----CAGATCAAGA	ATCAACTGG

Figure 3 For colony 3 only one mutant sequence was detected, indicating that these cells are likely only heterozygotic knockouts. In colony 6 two different mutant sequences were detected.

Phase 4: Second Round of Selection for Monoclonal Biallelic Knockout Clones

- Colony 6 was serially diluted into 96 well plates for monoclonal selection. Genomic DNA was extracted from these clones (i.e. 6a, 6b..), PCR amplified, cloned and sequenced.
- Of the colony 6 clones, sequencing showed that only clone 6a had a frameshift mutation in both alleles (**Figure 4**). A frameshift mutation disrupts the open reading frame, resulting in nonsense mediated decay of mRNA transcript.

Sequence Alignment	490	500	510
WT	CGGCAACCTCATGA	AACCAGATCAAGA	ATCAACTGG
sgRNA Sequence	-----CATGA	AACCAGATCAAGA	-----
Clone 6a-1	CGGCAACCTCAT	-----CAGATCAAGA	ATCAACTGG
Clone 6a-2	CGGCAACCTCATG	-----CCAGATCAAGA	ATCAACTGG
Clone 6b-1	CGGCAACCTCATGA	CCAGATCAAGA	ATCAACTGG
Clone 6b-2	CGGCAACCGAA	-----CCAGATCAAGA	ATCAACTGG
Clone 6d-1	CGGCAACCTC	-----CAGATCAAGA	ATCAACTGG
Clone 6d-2	CGGCAACCTCATGA	-----CCAGATCAAGA	ATCAACTGG

Figure 4 Clones 6a, 6b and 6d all showed biallelic editing. Only clone 6a had frame shift mutations in both alleles. No WT sequences were detected in all subclones.

Sequence Alignment	490	500	510
WT	CGGCAACCTCATGA	AACCAGATCAAGA	ATCAACTGG
sgRNA Sequence	-----CATGA	AACCAGATCAAGA	-----
Clone 6a-1	CGGCAACCTCAT	-----CAGATCAAGA	ATCAACTGG
Clone 6a-2	CGGCAACCTCATG	-----CCAGATCAAGA	ATCAACTGG
Clone 6a-3	CGGCAACCTCAT	-----CAGATCAAGA	ATCAACTGG
Clone 6a-4	CGGCAACCTCAT	-----CAGATCAAGA	ATCAACTGG
Clone 6a-5	CGGCAACCTCATG	-----CCAGATCAAGA	ATCAACTGG

Figure 5 Further sequencing of 6a confirmed biallelic knock-out. No WT sequences were detected.

- Further sequencing of clone 6a confirmed that only two mutant alleles were present, the 2 bp and 4 bp deletions, and that no WT or other mutations were detected (**Figure 5**).

Phase 4: Confirmation of Knockout by Next Generation Amplicon Sequencing

- With next generation sequencing hundreds of thousands of alleles can be sequenced at once, resulting in a more robust dataset. By contrast Sanger sequencing is only feasible for 1-100 clones and therefore it can miss a large proportion of the population.
- Next generation sequencing was performed at each stage of selection to evaluate knockout (**Figure 6**). Before editing, only WT sequences were observed. After the first round of selection colony 6 showed a mixture of edited (70%) and WT (30%) sequences. Finally after monoclonal selection, clone 6a showed only edited sequences with no WT alleles present.

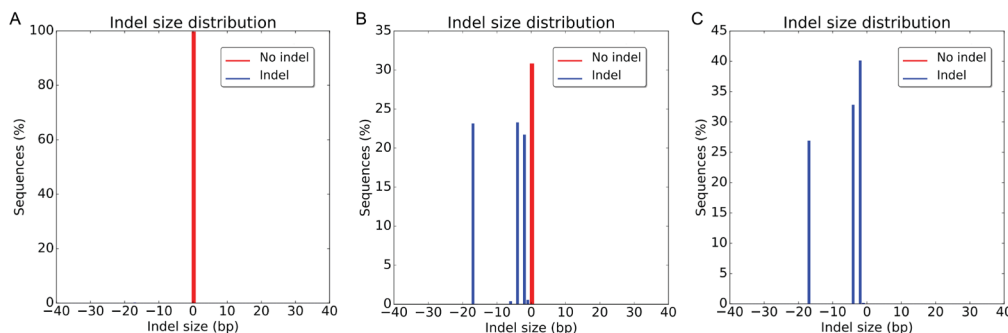


Figure 5 Next Generation Sequencing for CRISPR Knock-out screening. A) Before knock-out only WT sequences are detected. B) After Cas9 and sgRNA delivery, the first round of selection shows a mixed distribution of indel and WT sequences. C) After the second round of selection only knock-outs remain.