**Sample preparation & Next-generation Sequencing (NGS)**

**UC Berkeley & IGI**

**Lorena de Oñate (Ross Wilson’s Lab)**

**Cas9 RNP sample preparation**

Cas9 RNPs were prepared as described in Rouet *et al.* 2018: <https://pubs.acs.org/doi/10.1021/jacs.8b01551>

For triGalNAc-conjugated Cas9 protein, a simplified version of the ligand was used (in contrast to the version used in Rouet *et al.* 2018). This version was synthesized using (β-D-GalNAc-sp)3-NHC(O)-PEG5-COOH (Sussex Research) and PDPH (3-(2-pyridyldithio)propionyl hydrazide) (Fisher), generating a triGalNAc moiety bearing a pyridyl disulfide group, which was used for conjugation akin to that performed in Rouet 2018. RNPs were complexed with 30 molar equivalents of delivery peptide ppTG21 (sequence: GLFHALLHLLHSLWHLLLHA; from CPC Inc.) before use. 150 pmol of the complexed RNPs were administered every 600,000 hepatocytes (lot# HU2054). The sgRNA was synthesized as described in Rouet *et al.* 2018, and used spacer sequence CTTCTCTACACCCAGGGCAC to target exon 2 from the transthyretin (TTR) gene. Samples were prepared at a concentration of 8 μM and were diluted into 300 µL of cell culture media before administration by media exchange during 6 minutes. *Kiani Lab Info ?*

**PCR amplification of TTR locus for next-generation sequencing**

To determine and quantify the percentage of non-homologous end joining (NHEJ) in hepatocytes (lot# HU2054) after peptide mediated delivery of Cas9 ribonucleoprotein (RNP), cell samples were prepared as follows.

Genomic DNA (gDNA) was extracted 72 hours post-delivery using Quick Extract buffer (Lucigen) according to the manufacturer’s protocol. Briefly, after washing the cells with DPBS (-/-) twice, QE was added and incubated for 10 minutes at RT. The harvested cells were transferred to PCR tubes and incubated at the thermocycler at 65°C for 40 mins and 95°C for 20 mins. This is a longer incubation than the recommended by the manufacturer, which has demonstrated better results in our hands.

To generate PCR amplicons across the target sites, TTR locus-specific primers, forward 5’-GTGTCTGGAGGCAGAAACCA-3’ and reverse 5’-CAAGTCCTGTGGGAGGGTTC-3’ were designed using Primer Blast and the human reference genome sequence NC\_000018\_10, with the amplicon spanning a region of 376 bp around the predicted Cas9 cutting site in exon 2. Amplicon sequencing primers incorporate an adaptor or “stub” at the 5′ end, making the amplicon compatible with Illumina NGS library preparation.

Concentrations of genomic DNA samples were assessed using the Quant-iT Picogreen dsDNA assay kit (ThermoFisher P7589) following manufacturer’s instructions. The supplied DNA standard was diluted to the recommended concentration and analyzed alongside the samples. The samples were read in a TECAN fluorescence plate reader with an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

For PCR, 30–40 ng of gDNA input were used as template using the High Fidelity GXL Polymerase (TAKARA) and the following general thermocycler conditions: denaturing for 2 minutes at 95°C; then 32 cycles of 15 seconds at 95°C, primer annealing for 15 seconds at 60°C and amplification of 30 seconds at 68°C with final amplification of 2 minutes at 68°C. The resulting amplicons were analyzed by agarose gel electrophoresis. Then, the PCR products were purified by using silica magnetic beads (SPRI) prepared by the UC Berkeley Sequencing Facility comparable to AMPure XP (Beckman) and the products were quantified by NanoDrop spectrophotometer and adjusted to be at 10–20 ng/µL.

**High-throughput sequencing data analysis**

Purified PCR products were ligated to Illumina TruSeq adaptors and purified using SPRI beads. Libraries were prepared at the IGI Sequencing Facility by using a TruSeq Nano HT kit (Illumina) according to the manufacturer’s guidelines and using a final concentration of 1.36× Sample Purification Beads (Ilumina) following end repair for further size selection and followed by high-throughput sequencing with the MiSeq platform.

Samples were deep sequenced on an Illumina Miseq at 300 bp paired-end reads to a depth of ≥ 10,000 reads. As described previously (1), editing outcomes were assessed using Cortado v1.0 (<https://github.com/staciawyman/cortado>). Sequences were adaptor-trimmed and then joined before performing a global alignment between reads and the reference sequence using NEEDLE (2). Indel rates were calculated as follows: any reads in which an insertion or deletion overlaps the cut site or occurs within a 3 base pair window around the cut site, normalized to the total number of aligned reads.

1. Wienert B, Wyman SK, Richardson CD, et al. Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. *Science*. 2019;364(6437):286-289. doi:10.1126/science.aav9023
2. Li W, Cowley A, Uludag M, et al. The EMBL-EBI bioinformatics web and programmatic tools framework. *Nucleic Acids Res*. 2015;43(W1):W580-W584. doi:10.1093/nar/gkv279
3. Rouet R, Thuma BA, Roy MD, Lintner NG, Rubitski DM, Finley JE, Wisniewska HM, Mendonsa R, Hirsh A, de Oñate L, Compte Barrón J, McLellan TJ, Bellenger J, Feng X, Varghese A, Chrunyk BA, Borzilleri K, Hesp KD, Zhou K, Ma N, Tu M, Dullea R, McClure KF, Wilson RC, Liras S, Mascitti V, Doudna JA. Receptor-Mediated Delivery of CRISPR-Cas9 Endonuclease for Cell-Type-Specific Gene Editing. J Am Chem Soc. 2018 May 30;140(21):6596-6603. doi: 10.1021/jacs.8b01551. Epub 2018 May 18. PMID: 29668265; PMCID: PMC6002863.