**Materials and Methods:**

The *in vivo* editing experiments in this publication were performed using a technique called nucleofection, which introduces functional Cas9 protein and the targeting guide RNA into cells by electroporation. Results from our lab and others (PMIDs 25497837, 26789497 and 24696461) have shown that this technique supports extremely high frequencies of genome cutting and, when donor DNA is included in the nucleofection reaction, extremely high frequencies of homology-directed repair. Below, we have described the reagents and protocols that we used to perform nucleofection reactions in HEK293 and K562 human cell lines. We encourage any labs performing genome editing experiments to try nucleofection as (in our hands) this technique supports gene disruption or gene replacement frequencies that are an order of magnitude greater than transfection approaches. Moreover, the cost of reagents is relatively modest, requiring expressed Cas9 protein (see Cas9 species/variant), transcribed sgRNA, and optional donor DNA (see sequences in Validated editing reagents).

**Cell Types:**

Human Embryonic Kidney (HEK293) Cells, Human Myelogenous Leukemia (K562) Cells

**Target Genes:**

BFP transgenic reporter (Addgene 71825), CCR5, CXCR4, EMX1

**Cas9 species/variant**

*S. Pyogenes* Cas9 with C-terminal 2xNLS (Addgene 69090)

*S. Pyogenes* nCas9 (D10A) with C-terminal 2xNLS (Addgene 71822)

*S. Pyogenes* nCas9 (H840A) with C-terminal 2xNLS (Addgene 71823)

*S. Pyogenes* dCas9 (D10A, H840A) with C-terminal 2xNLS (Addgene 71824)

**Delivery method**

100 pmoles of Cas9-2NLS (or variants) was diluted to a final volume of 5uL with Cas9 buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM MgCl2, 10% glycerol and 1 mM TCEP) and mixed slowly into 5uL of Cas9 buffer containing 120 pmoles of sgRNA. The resulting mixture was incubated for ten minutes at RT to allow RNP formation. 2E+05 cells were harvested, washed once in PBS, and resuspended in 20uL of nucleofection buffer (Lonza, Basel, Switzerland). 10uL of RNP mixture, 4.5 uL of N-oligo, and cell suspension were combined in a Lonza 4d strip nucleocuvette. Reaction mixtures were electroporated, incubated in the nucleocuvette at RT for ten minutes, and transferred to culture dishes containing pre-warmed media (**dx.doi.org/10.17504/protocols.io.dm649d**). Nucleofection buffer and electroporation conditions were the following for each cell line: HEK293 in SF with DS-150, K562 in SF with FF-120.

**Publication**

doi:10.1038/nbt.3481

**Validated Editing Reagents**

|  |  |  |  |
| --- | --- | --- | --- |
| **Editing Locus** | **Target Site (PAM in bold)** | **Guide (protospacer in CAPS)** | **Donor** |
| BFP (Cas9) | **CCC**ATGGCGTGCAGTGCTTCAGC | ggatcctaatacgactcactatagGCTGAAGCACTGCACGCCATgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt | GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGA |
| BFP (dCas9 Tiling) | **CCT**GAAGTTCATCTGCACCACCG | ggatcctaatacgactcactatagCGGTGGTGCAGATGAACTTCgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt | TGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACGCTGAAGTTCATCTGCACCACCGGCAAGCTGCCGGTGCCCTGGCCCACCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACGACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAG |
|  | **CCC**ATGGCGTGCAGTGCTTCAGC | ggatcctaatacgactcactatagGCTGAAGCACTGCACGCCATgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt |  |
|  | **CCA**CATGAAGCAGCACGACTTCT | ggatcctaatacgactcactatagAGAAGTCGTGCTGCTTCATGgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt |  |
| CCR5 | TGACATCAATTATTATACAT**CGG** | ggatcctaatacgactcactatagTGACATCAATTATTATACATgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt | ACAAAACCAAAGATGAACACCAGTGAGTAGAGCGGAGGCAGGAGGCGGGCTGCGATTTGCTTCACATTGATTTTTTGGCAGGGCTCacATGTATAATAATTGATGTCATAGATTGGACTTGACACTT |
| CXCR4 | **CCT**CCTCTTTGTCATCACGCTTC | ggatcctaatacgactcactatagAAGCGTGATGACAAAGAGGgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt | ATGGATTGGTCATCCTGGTCATGGGTTACCAGAAGAAACTGAGAAGCATGACGGACAAGTACAGGCTGCACCTGTCAGTGGCCGACATGTTCTTTGTCATCACGCTTCCCTTCTGGGCAGTTGATGC |
| EMX1 | CGATGTCACCTCCAATGACT**AGG** | ggatcctaatacgactcactatagCGATGTCACCTCCAATGACTgttttagagctagaaatagcaagttaaaataggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttttt | AGTGGCCAGAGTCCAGCTTGGGCCCACGCAGGGGCCTGGCCAGCAGCAAGCAGCACTCTGCCCTCGTGGGTTTGTGGTTGCCCACACATGTCATTGGAGGTGACATCGATGTCCTCCCCATTGGCCT |