

CRISPR/Cas9 Systems Targeting Beta-globin and CCR5 Genes

Have Substantial Off-target Activity

Thomas J. Cradick, Eli J. Fine, Christopher J. Antico and Gang Bao

SUPPLEMENTARY INFORMATION

Supplementary Figure S1. Sequencing results of on- and off-target cleavage induced by CRISPR/Cas9.

Supplementary Figure S2. A comparison of on- and off-target mutation rates.

Supplementary Figure S3. On- and off-target mutation rates of CRISPR/Cas9 systems targeting *CCR5*.

Supplementary Figure S4. Transfection dosage variably affects on- and off-target mutation rates.

Supplementary Figure S5. Quantitative PCR determination of the percentage of *HBD-HBB* chromosomal deletions.

Supplementary Table S1. Sequence of primers used to amplify endogenous loci for the T7E1 assay, sequencing and quantitative PCR.

Supplementary Figure S1. Sequencing results of on- and off-target cleavage induced by CRISPR/Cas9. (a) R-02 targeting *HBB*. (b) R-02 off-target site 2, *GRIN3A*. (c) R-01 targeting *HBB* and *HBD*. (d) R-04 targeting *HBB* and *HBD*. (e) R-08 targeting *HBB*. (f) R-26 targeting *CCR5*. (g) R-27 targeting *CCR5*. (h) R-30 cleavage at *CCR2* and chromosomal deletions between *HBD* and *HBB*. HEK-293T cells were transfected with each CRISPR construct, and their genomic DNA harvested after three days in culture. Target loci were amplified, cloned and Sanger sequenced. Sequences were aligned to the reference gene, listed above the alignment, with the guide strand, the number of clones with indels, the total number of clones and percentage with indels. The alignment includes the reference gene and guide strand with mismatches boxed. The first column lists the number of times each read occurred and indel size change in bp. Unmodified reads are indicated by "WT". Yellow highlights mutations and insertions, with deletions (:) highlighted grey. For clarity, the nucleotides A, C, T and G are shown in green, blue, red and black respectively.

Supplementary Figure 1

Supplementary Figure 1A

R-02 HBB 60/80 = 75%

```

-45 TTCATCCACGTTCA:.....GGTGA
-45 TTCATCCACGTTCA:.....GTGA
-23 TTCATCCACGTTCA:.....AGACTTCTCCTCAGGAGTCAGGTGCA
2x -22 TTCATCCACAT:.....AACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-19 TTCATCCACGTTACCTTGC:.....AGACTTCTCCTCAGGAGTCAGGTGCA
-17 TTCATCCACGTTACCTTGCCCCACAGGGCAG:.....TCAGGAGTCAGGTGCA
-16 TTCATCCACGTTACCT:.....AACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-11 TTCATCCACGTTACCTTGCCCCA:.....CGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-10 TTCATCCACGTTACCTTGCCC:.....TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
2x -9 TCCATCCACGTTACCTTGCCCCACA:.....CGGCAGACTTCTCCTCAGGAGTCAGGTGCA
15x -9 TTCATCCACGTTACCTTGCCCCACAG:.....GGCAGACTTCTCCTCAGGAGTCAGGTGCA
-9 TTCATCCACGTTACCTTGCCCCACAGGG:.....TAGACTTCTCCTCAGGAGTCAGGTGCA
-8 TTCATCCACGTTACCTTGCCCCACAGG:.....GCAGGACTTCTCCTCAGGAGTCAGGTGCA
3x -7 TTCATCCACGTTACCTTGCCCCAC:.....TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
2x -6 TTCATCCACGTTACCTTGCCCCACAGGGCA:.....GCAGACTTCTCCTCAGGAGTCAGGTGCA
-6 TTCATCCACGTTACCTTGCCCCACA:.....TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-5 TTCATCCACGTTACCTTGCCCCACAGGGCA:.....GGCAGACTTCTCCTCAGGAGTCAGGTGCA
-5 TTCATCCACGTTACCTTGCCCCACA:.....GTAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
2x -3 TTCATCCACGTTACCTTGCCCCACAGGG:.....TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-2 TTCATCCACGTTACCTTGCCCCACAG:.....CAGTAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
2x -2 TTCATCCACGTTACCTTGCCCCACAGGGC:.....TAACGGCAAACCTTCTCCTCAGGAGTCAGGTGCA
-2 TTCATCCACGTTACCTTGCCCCACAGGGCA:.....AACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
3x -1 TTCATCCACGTTACCTTGCCCCACAGGGCAG:.....AACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
-1 TTCATCCACGTTACCTTGCCCCACAGGGCA:.....TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
3x TTCATCCACGTTACCTTGCCCCACAGGGCA:.....TTGACAGCAGACTTCTCCTCAGGAGTCAGGTGCA
HBB TTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
20x WT TTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAGGTGCA
R-02 GTTGCCCCACAGGGCAGTAANGG
2x +1 TTCATCCACGTTACCTTGCCCCACAGGGCAGTTAACGGCAGACTTCTCCTCAGGAGTCAGGTGTC
2x +1 TTCATCCACGTTACCTTGCCCCACAGGGCAGATAACGGCAGACTTCTCCTCAGGAGTCAGGTGTC
+1 TTCATCCACGTTACCTTGCCCCACAGGGCAAGTAACGGCAGACTTCTCCTCAGGAGTCAGGTGTC
2x +1 TTCATCCACGTTACCTTGCCCCACAGGGCAGGTAACGGCAGACTTCTCCTCAGGAGTCAGGTGTC
2x +2 TTCATCCACGTTACCTTGCCCCACAGGGCAGAGTAACGGCAGACTTCTCCTCAGGAGTCAGGTG
+3 TTCATCCACGTTACCTTGCCCCACAGGGCAATATTAAACGGCAGACTTCTCCTCAGGAGTCAGGT

```

Supplementary Figure 1B

R-02 Off-target-2 GRIN3A 23/30 = 77%

```

-134 AGTCAGAGCAGTGCTTCAGCCCCACAGGGGCTG:.....
-34 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCCCTGT:.....
-14 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAG:.....CTCTAAATACCAGATTCCC
16x -9 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAG:.....CCTTCCTCTAAATACCAGATTCCC
-1 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCA:.....TAAGGGCAGCCTTCTCTAAATACCAGATTCCC
GRIN3A AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAGTAAGGGCAGCCTTCTCTAAATACCAGATTCCC
7x WT AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAGTAAGGGCAGCCTTCTCTAAATACCAGATTCCC
R-02 GTTGCCCCACAGGGCAGTAANGG
+1 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAGTTAAGGGCAGCCTTCTCTAAATACCAGATTCC
+1 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAGCTAAGGGCAGCCTTCTCTAAATACCAGATTCC
+1 AGTCAGAGCAGTGCTTCAGCCCCACAGGGCAGTATAAGGGCAGCCTTCTCTAAATACCAGATTCC

```

Supplementary Figure 1C

R-01 HBB 8/12=67%

```

-111 GCCCTGTGGGGCAAGGTGAACGTGGAT:.....
-15  NCCCTGAGGGGCAAGGTGAAC:.....GGTGAGGCCCTGGGCAGGTTGGTATCAAG
-13  GCCCTGTGGGGCAAGGTGA:.....TGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAG
2x   -2  GCCCTGTGGGGCAAGGTGAACGTGGATGAAG:TTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAG
      HBB GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAG
4x   WT  GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAG
      R-01          GTGAACGTGGATGAAGTTGGNNG
2x   +1  GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAA
      +7  GCCCTGTGGGGCAAGGTGAACGTGGAACGTGGATGAGTTGGTGGTGAGGCCCTGGGCAGGTTGG

```

R-01 HBD 3/10=30%

```

-248 :.....TGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGG
0     GCCCTGTGGGGCAAAGTGAACGTGGATGCAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGG
      HBD GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGG
7x   WT  GCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGG
      R-01          GTGAACGTGGATGAAGTTGGNNG
2x   +1  GCCCTGTGGGGCAAAGTGAACGTGGATGCAGTTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAG

```

Supplementary Figure 1D

R-04 HBB =7/15=47%

```

-10  CACCACCAACTTTCATCCACGTTCCAC:.....AGGGCAGTAACGGCAGACTTCTCCTCAGGA
-9   CACCACCAACTTTCATCCACGTTCCAC:.....AGGGCAGTAACGGCAGACTTCTCCTCANGA
-10  CACCACCAACTTTCATCCACGTTACCTTGCC:.....GTAACGGCAGACTTCTCCTCAGGA
-7   CACCACCAACTTTCATCCACGTTACCTTGCCC:.....CAGTAACGGCAGACTTCTCCTCAGGA
-3   CACCACCAACTTTCATCCACGTTACCTTGCCCC:.....GGGCAGTAACGGCAGACTTCTCCTCAGGA
-2   CACCACCAACTTTCATCCACGTTACCTTGCC:ACAGGGCAGTAACGGCAGACTTCTCCTCAGGA
8x   WT  CACCACCAACTTTCATCCACGTTACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGA
      HBB CACCACCAACTTTCATCCACGTTACCTTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGA
      R-04          GCACGTTACCTTGCCCCACNNG
      +6  CACCACCAACTTTCATCCACGTTACCTTGCCCCGTGTCATCACAGGGCAGTAACGGCAGACTTCTCC

```

Supplementary Figure 1E

R-08 HBB 10/20=50%

```

-27  GTCTGCCGTTACTGT:.....AAGTTGGTGGTGAGGCCCTGGGCAG
-16  GTCTGCCGTTACTGCCCTGTGGGGCA:.....AGTTGGTGGTGAGGCCCTGGGCAG
-12  GTCTGCCGTTACTGCCCTGTG:.....ACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG
-7   GTCTGCCGTTACTGCCCTGTGGGGCA:.....CGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG
2x   -1  GTCTGCCGTTACTGCCCTGTGGGGCAAGGTGA:CGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG
      HBB GTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG
10x  WT  GTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG
      R-08          CTGTGGGGCAAGGTGAACGNGG
4x   +1  GTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG

```

Supplementary Figure 1F

R-26 CCR5 16/21 = 76%

```

-23 GTCCCCCTTCTGGGCTC:.....:TTTGGAAATACAATGTGTCAACTCTT
-13 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCA:.....:TCCAATGTGTCAACTCTT
-9 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCC:.....:GTGGGAATACAATGTGTCAACTCTT
3x -8 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGTGGG:.....:AAATACAATGTGTCAACTCTT
3x -7 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGTGGG:.....:AAATACAATGTGTCAACTCTT
2x -7 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGTG:.....:GGAAATACAATGTGTCAACTCTT
-5 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGCT:.....:TTGGAAATACAATGTGTCAACTCTT
-8 GT:.....:GGGCTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
-5 GTCCCCCTT:.....:CTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
-2 GTCCCCCTT:GGGCTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
-1 GTCCCCCTTCT:GGGCTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
CCR5 GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
5x WT GTCCCCCTTCTGGGCTCAGTATGCTGCCGCCAGTGGGACTTTGGAAATACAATGTGTCAACTCTT
R-26 GTGCCGCCAGTGGGACTTNGG

```

Supplementary Figure 1G

R-27 CCR5 7/9 = 78%

```

-30 AAAG:.....:AAGGGGACAGTAAGAAGGAAAAACAGGTCAG
2x -14 AAAGTCCCACTGGGCGGCAG:.....:AAGGGGACAGTAAGAAGGAAAAACAGGTCAG
-13 AAAGTCCCACTGGGCGGCAG:.....:AAGGGGACAGTAAGAAGGAAAAACAGGTCAG
-2 AAAGTCCCACTGGGCGGCAGCATAGTGAGC:AGAAGGGGACAGTAAGAAGGAAAAACAGGTCAG
CCR5 AAAGTCCCACTGGGCGGCAGCATAGTGAGCCCAAGAAGGGGACAGTAAGAAGGAAAAACAGGTCAG
2x WT AAAGTCCCACTGGGCGGCAGCATAGTGAGCCCAAGAAGGGGACAGTAAGAAGGAAAAACAGGTCAG
R-27 GGAGCATAGTGAGCCAGANGG
2x +1 AAAGTCCCACTGGGCGGCAGCATAGTGAGCCCAAGAAGGGGACAGTAAGAAGGAAAAACAGGTCAG

```

Supplementary Figure 1H

R-30 CCR2 Off-target 9/43 = 21%

```

-7 GATGAACACCAGCGAGTAGAGCGGAGGCAGGA:.....:CCCAATTTGCTTCACGTCAAATTTAT
-5 GATGAACACCAGCGAGTAGAGCGGGGGCAG:.....:GGCCTCCAATTTGCTTCACGTCAAATTTAT
-5 GATGAACACCAGCGAGTAGAGCGGAG:.....:AGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
-4 GATGAACACCAGCGAGTAGAGCGGAGG:.....:AGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
-1 GATGAACACCAGCGAGTAGAGCGGAGGCAG:AGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
-1 GATGAACACCAGCGAGTAGAGCGGAGGCAGGA:TTGGGCCCCAATTTGCTTCACGTCAAATTTAT
CCR2 GATGAACACCAGCGAGTAGAGCGGAGGCAGGAGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
34x WT GATGAACACCAGCGAGTAGAGCGGAGGCAGGAGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
R-30 GTAGAGCGGAGGCAGGAGGCGCNGG
2X +1 GATGAACACCAGCGAGTAGAGCGGAGGCAGGAAGTTGGGCCCCAATTTGCTTCACGTCAAATTTA
+2 GATGAACACCAGCGAGTAGAGCGGAGGCAGGAGCAGTTGGGCCCCAATTTGCTTCACGTCAAAT

```

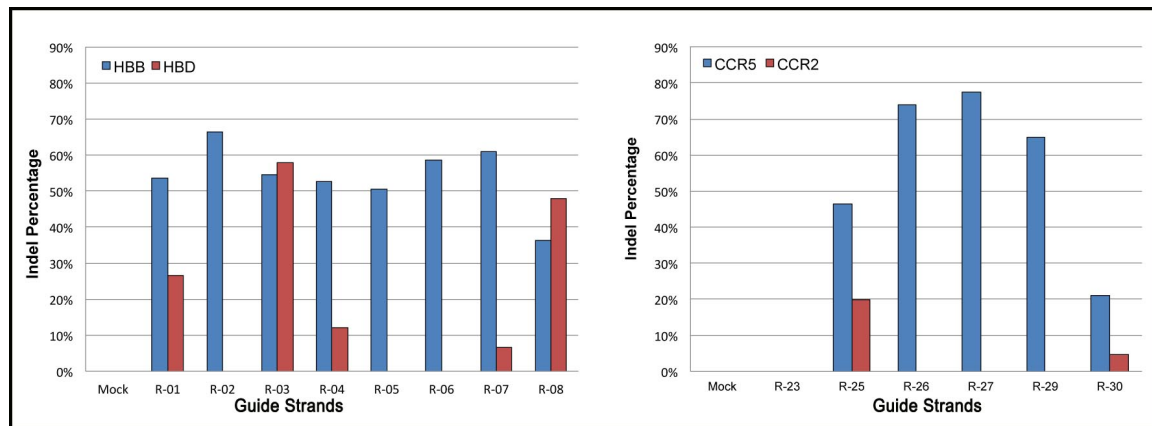
R-30 CCR5 to CCR2

```

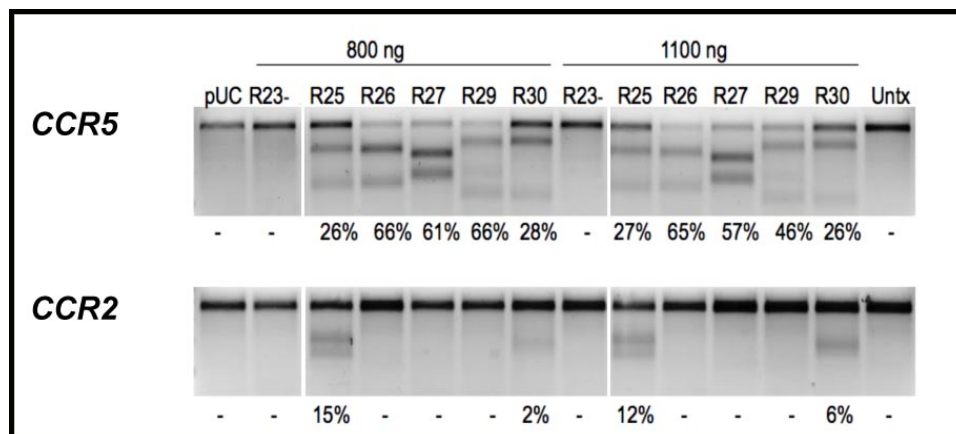
-11 GATGAACACCAGTGAGTAGAGCGGAGGCAGG:.....:AATTTGCTTCACGTCAAATTTAT
-11 GATGAACACCAGTGAGTAGA:.....:AGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
-9 GATGAACACCAGTGAGTAGAGCGGAGGCAGGA:.....:CAATTTGCTTCACGTCAAATTTAT
-7 GATGAACACCAGTGAGTAGAGCGGAGG:.....:TGGGCCCCAATTTGCTTCACGTCAAATTTAT
-2 GATGAACACCAGTGAGTAGAGCGGAGGC:GAGTTGGGCCCCAATTTGCTTACGTCAAATTTAT
2X -1 GATGAACACCAGTGAGTAGAGCGGAGGCAG:AGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
2X GATGAACACCAGTGAGTAGAGCGGAGGCAGGAGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
CCR5/2 GATGAACACCAGTGAGTAGAGCGGAGGCAGGAGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT
WT GATGAACACCAGTGAGTAGAGCGGAGGCAGGAGTTGGGCCCCAATTTGCTTCACGTCAAATTTAT

```

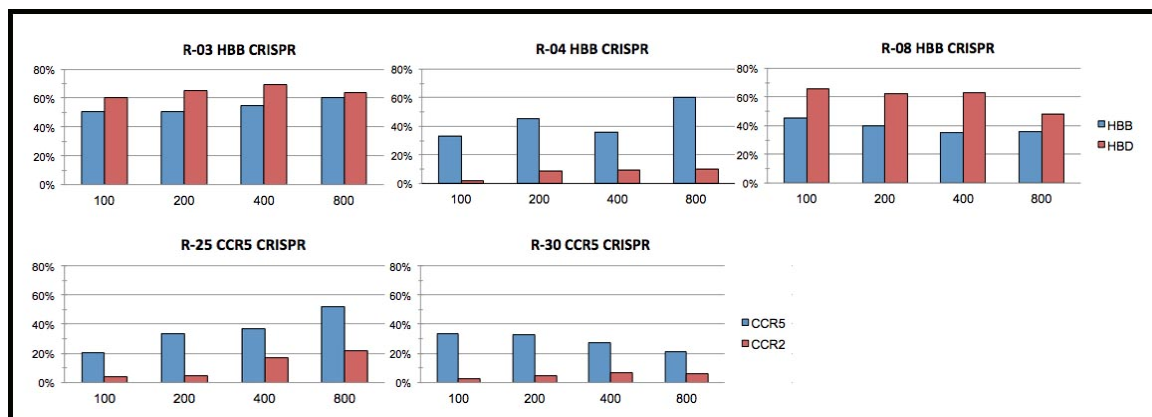
Supplementary Figure S2. A comparison of on- and off-target mutation rates. HEK-293 cells were transfected in triplicate, their genomic DNA harvested, the target (*HBB* or *CCR5*) and off-target loci (*HBD* or *CCR2*) were amplified and the indel percentage determined using the T7E1 mutation detection assay. Indel percentages are plotted for the on- and off-target site for each guide strand.



Supplementary Figure S3. On- and off-target mutation rates of CRISPR/Cas9 systems targeting *CCR5*. The *CCR5* samples were transfected at two concentrations, 800 ng and 1100 ng and the mutation rates were quantified using T7E1. The indel percentages determined using ImageJ are listed below lanes having detectable amount. R-23 targeting *CCR5* was used a negative control for *CCR5*.

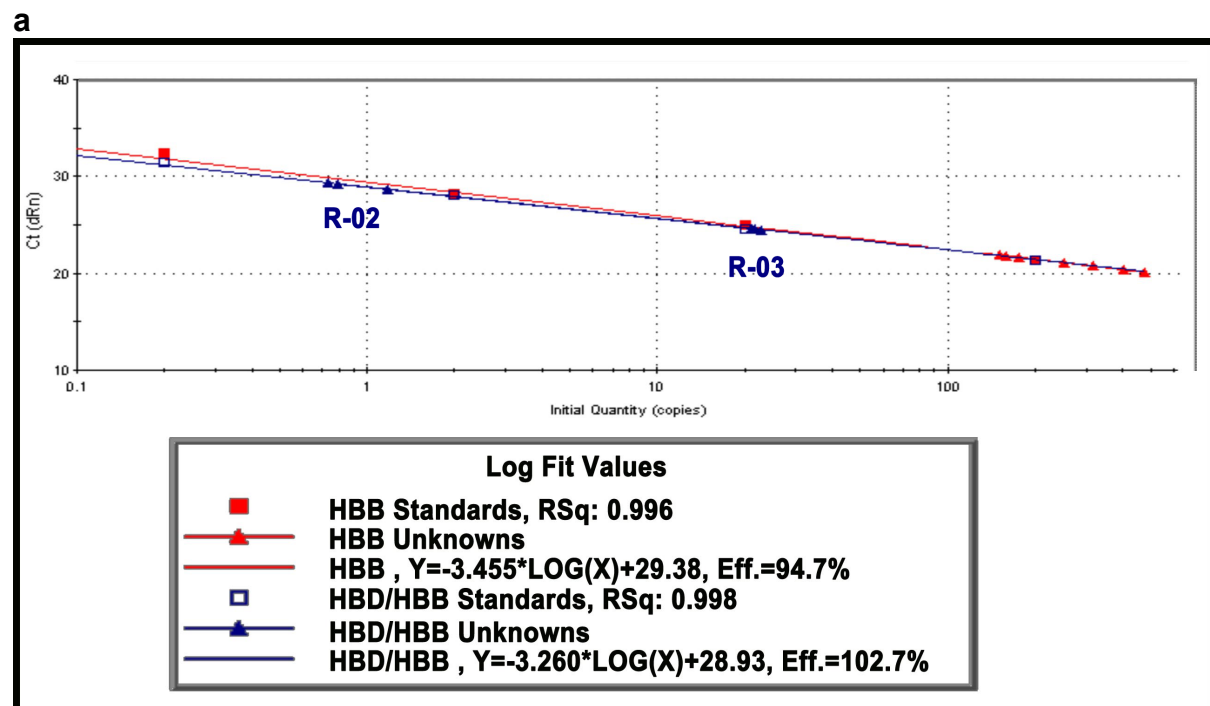


Supplementary Figure S4. Transfection dosage variably affects on- and off-target mutation rates. Mutation detection assay results for transfections using different doses of CRISPR plasmids. HEK-293 cells were transfected with 100 to 800 ng of CRISPR plasmids containing different guide strands. The genomic DNA was harvested and the target and off-target loci amplified. The PCR products were digested using T7E1 and loaded on agarose gels for quantitation using ImageJ. The gels for R-03 and R-25 are shown in Figures 1B and 3B, respectively. Lower doses of R-04 gave lower on- and off-target mutagenesis and R-25 had lower mutagenesis at both sites, whereas R-30 had low off-target mutagenesis at each dose. R-03 and R-08 had significant on- and off-target activity at each of these dosages. For R-03 and R-08 the ratio of on- to off-target activity did not improve with lower doses, as it did at 100ng for the other guide strands.



Supplementary Figure S5. Quantitative PCR determination of the percentage of *HBD-HBB* chromosomal deletions. HEK-293 cells were transfected in triplicate with CRISPR plasmids containing guide strands R-02 or R-03, or mock transfected cells, as described in Methods and reported in Supplementary Figure S3. Genomic DNA was harvested using QuickExtract (EpiCentre), per manufacturer's protocol. Amplification reactions contained 1 ul of genomic DNA added to mastermix aliquots containing: 0.1 ul of each 10 uM primer, 3.8 ul of water and 5 ul of iTaq Universal SYBR Green 2x Supermix. The reactions were analysed on an Mx3005P qPCR System (Stratagene) using MxPro qPCR software. As the genomic DNA could not be normalized, the total amount of *HBB* and the amount of *HBD* to *HBB* deletions were measured to determine the percentage of chromosomal deletions. Total *HBB* was measured using primers HBB-308R and HBB-mid99 that generated a 99 bp product from unmodified *HBB* or from chromosomal DNA with *HBD* to *HBB* deletions, as the primers bind outside the cleavage site. The *HBD-HBB* chromosomal deletion was measured using primers HBB-308R and HBD-520F and generates a 225 bp product that spans the cleavage site. The *HBB* product was seen in mock transfections, as *HBB* was unmodified. Mock transfection DNA did not amplify using HBB-308R and HBD-520F, indicating a lack of these chromosomal deletions. No template controls for each primer set were negative.

(a) Standard curves were made using serial dilutions of cloned *HBD-HBB* deletion fragment, so that the standard curves of both sets of primers can be compared. Quantities were very similar across this standard curve using either the *HBB* pair of primers or the *HBD-HBB* pair of primers, which allowed comparison of the total amount of *HBB* and the amount of *HBD* to *HBB* deletions. The groupings of three HBD/HBB samples for R-02 and R-03 are labelled.



(b) Genomic DNA from the cells transfected with guide strand R-03 contained *HBD-HBB* chromosomal deletions equal to 12.6% of the copies of total *HBB*. This was compared to genomic DNA from the cells transfected with guide strand R-02, which had higher HBB cleavage, but low HBD cleavage. The R-02 treated genomic DNA contained *HBD-HBB* chromosomal deletions equal to 0.4% of the copies of total *HBB*.

b

	Total HBB	HBD-HBB	HBD-HBB/ Total HBB	AVG	ST DEV
R-02a	251.80	0.7	0.3%		
R-02b	318.20	1.2	0.4%	0.4%	0.001
R-02c	159.20	0.8	0.5%		
R-03a	176.20	21.1	11.9%		
R-03b	201.00	22.8	11.4%	12.6%	0.016
R-03c	151.20	21.8	14.4%		
mock	479.80	0.0	0.0%		
mock	404.90	0.0	0.0%	0.0%	0.000
mock	175.60	0.0	0.0%		

Supplementary Table

Supplementary Table S1. Sequence of primers used to amplify endogenous loci for the T7E1 assay, sequencing and quantitative PCR.

Gene	Primer Sequence
CCR5 - F	GCACAGGGTGGAACAAGATGG
CCR5 - R	GACCACCCCAAAGGTGACCGT
CCR2 - F	TTGAACAAGGACGCATTTCCCCAG
CCR2 - R	CAAAGACCCACTCATTTGCAGCAG
HBB - F	CCAATAGGCAGAGAGAGTCAGTG
HBB - R	AGCCAGGGCTGGGCATAAAAG
HBD - F	GAGGTTGTCCAGGTGAGCCAGGCCATCAC
HBD - R	CTGCTGAAAGAGATGCGGTGGGGAGATATGTA
HBD-521F	AAGGCAGGGCAGAGTCGA
HBB-308R	CACATGCCCAGTTTCTATTGGT
HBB-mid99	GCAAGGTGAACGTGGATGA