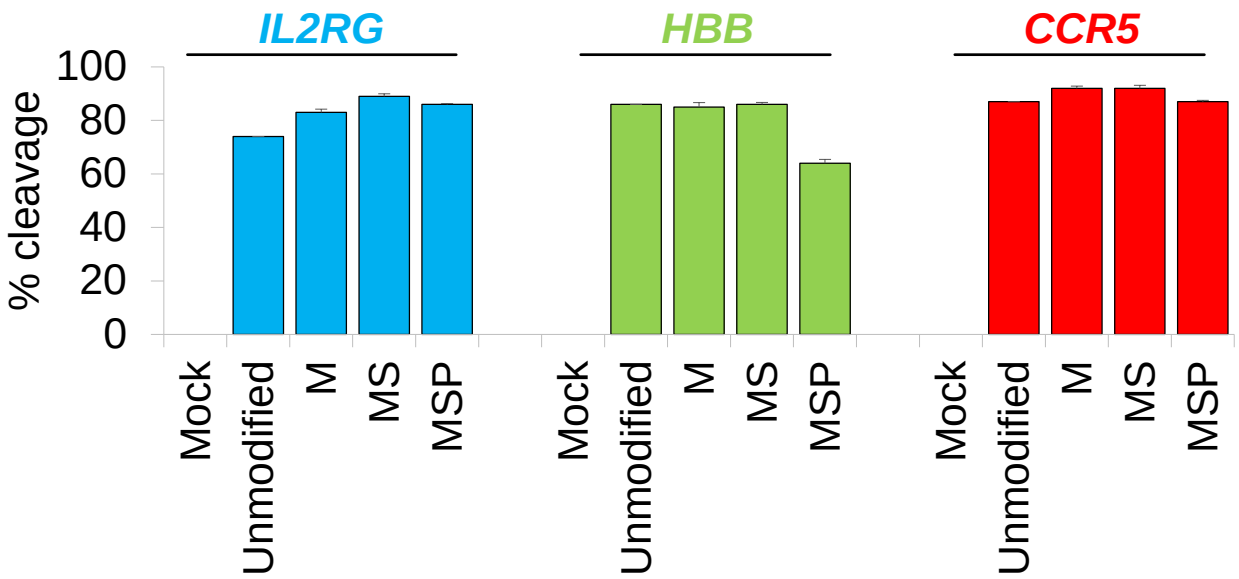

Supplementary Fig. 1. Cas9 cleavage of dsDNA targets directed by chemically modified sgRNAs *in vitro*. Cas9 cleavage of dsDNA targets directed by chemically modified sgRNAs *in vitro*. Bars indicate percent yield of cleavage products of target DNA fragments (see Supplementary Fig. 2) treated with Cas9 protein and sgRNA. Average values + s.e.m. for three independent syntheses of each sgRNA are shown.

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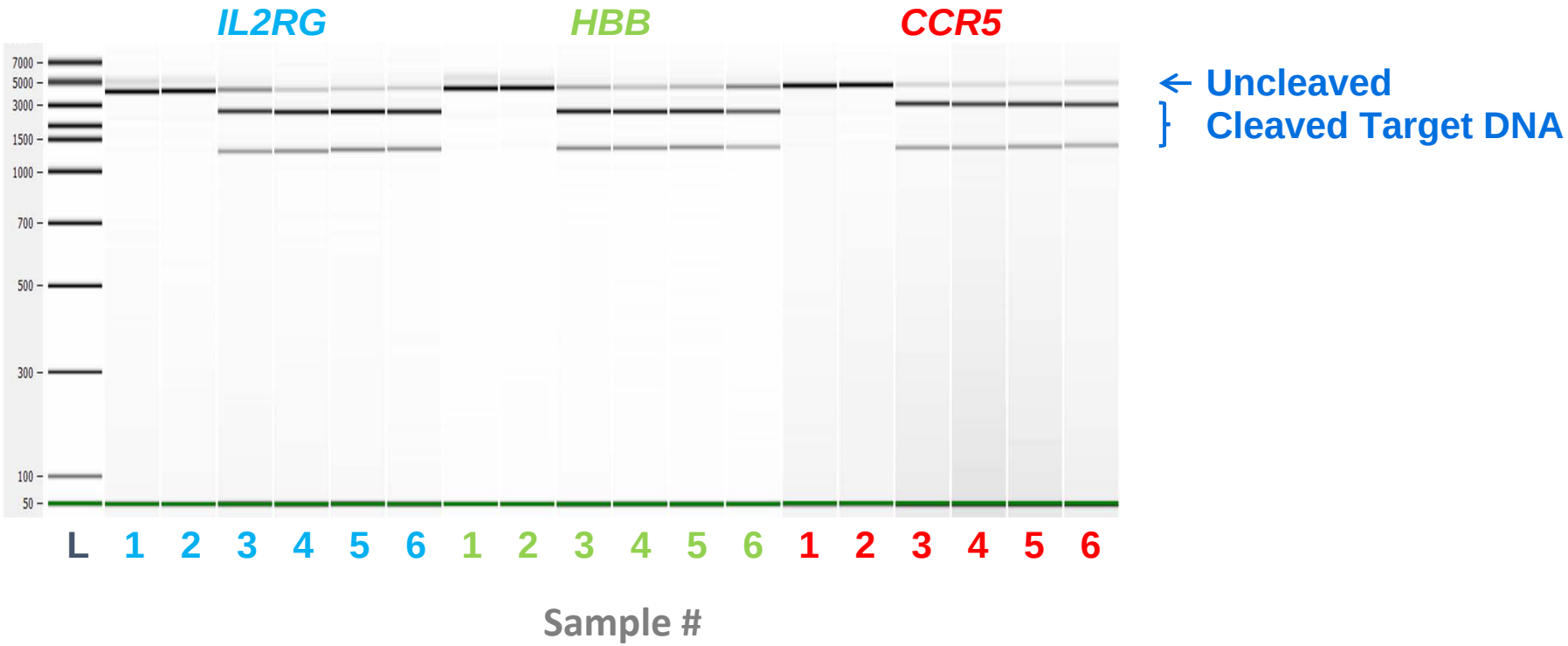
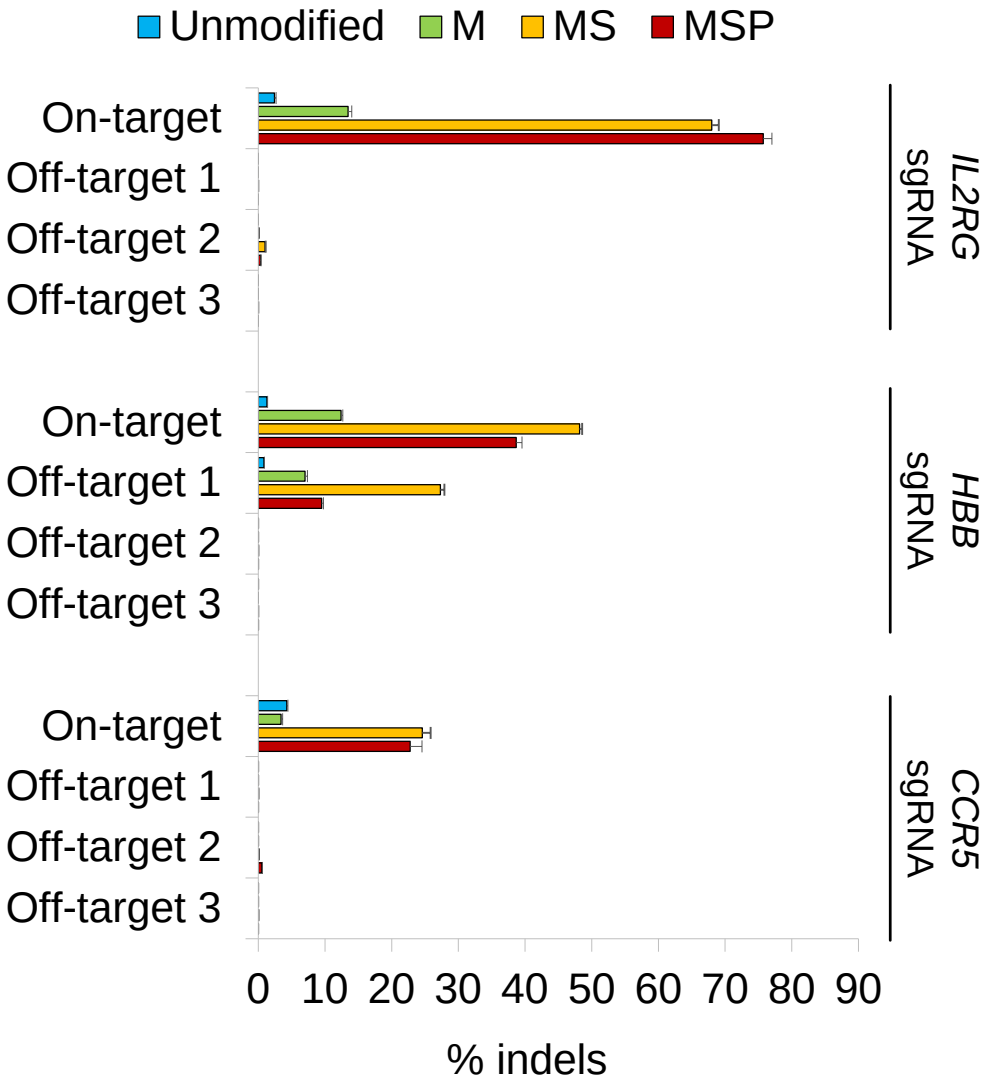


Supplementary Fig. 2. Cas9 cleavage of dsDNA targets directed by chemically modified sgRNAs *in vitro*. Cleavage products from biochemical cleavage of dsDNA targets were assayed on DNA 7500 LabChips on a Bioanalyzer 2200. Representative gels are shown for each target, and additional replicates are included in the results plotted in Supplementary Fig. 1.

Sample numbers:
L. Ladder
1. Unmodified sgRNA + target DNA (mock-treated minus Cas9)
2. Target DNA + Cas9 protein (mock-treated minus sgRNA)
3. Unmodified sgRNA + target DNA + Cas9 protein
4. M sgRNA + target DNA + Cas9 protein
5. MS sgRNA + target DNA + Cas9 protein
6. MSP sgRNA + target DNA + Cas9 protein

Nature Biotechnology: doi:10.1038/nbt.3290



Supplementary Fig. 3. Specificity of targeted cleavage mediated by synthetic sgRNAs. Target specificity was assessed as in Fig. 1e using Illumina deep sequencing, but with the samples from Fig. 1c nucleofected with 1 µg sgRNA. Indel frequencies were measured by deep sequencing of PCR amplicons from the three bioinformatically predicted off-target loci for each sgRNA. Bars represent average values + s.e.m., n=3. See also Supplementary Table 2 for a table with the indel percentages.

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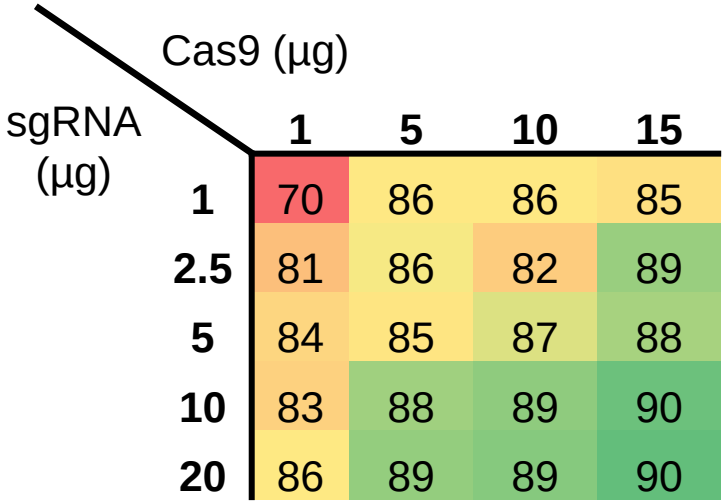
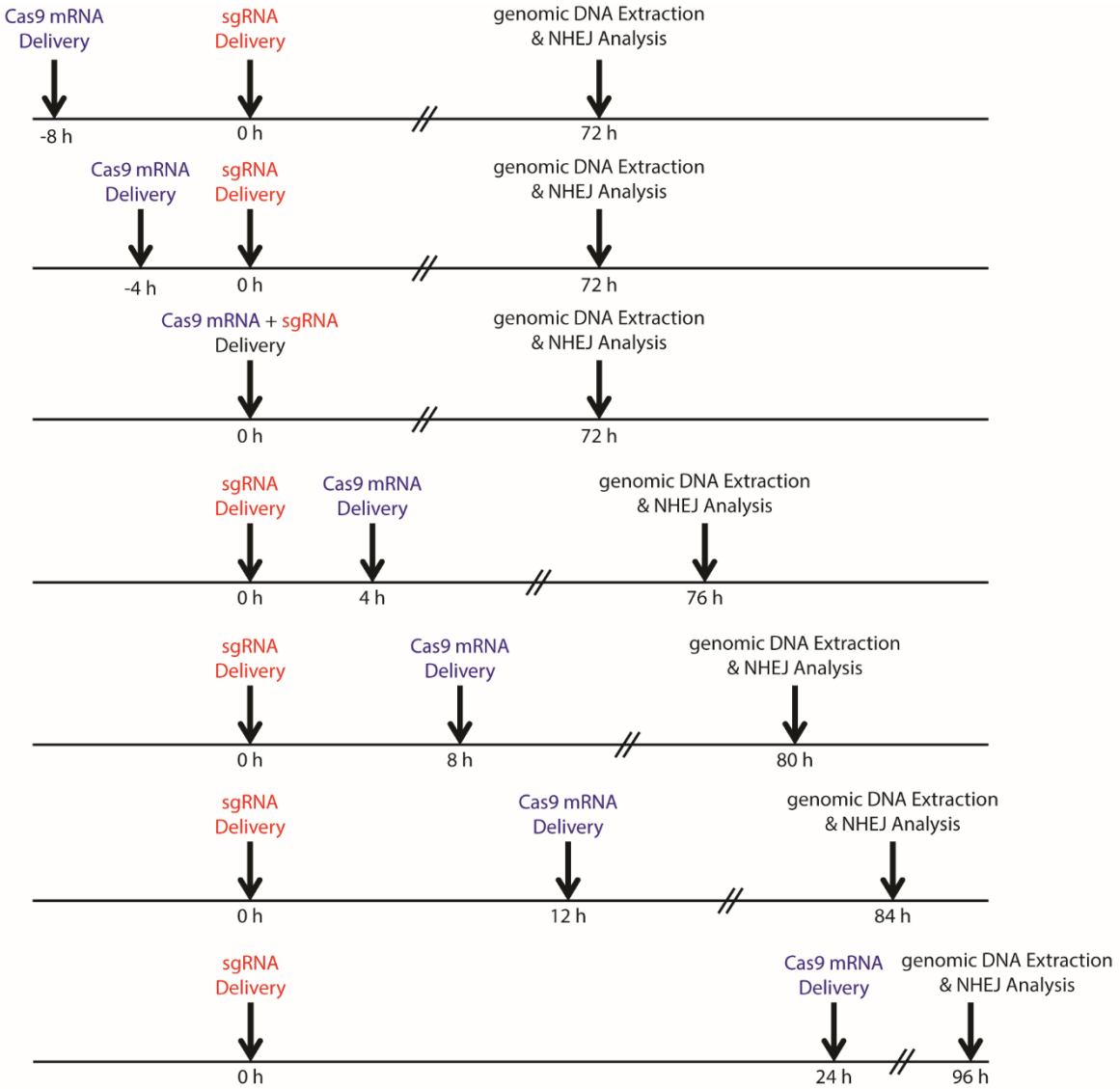


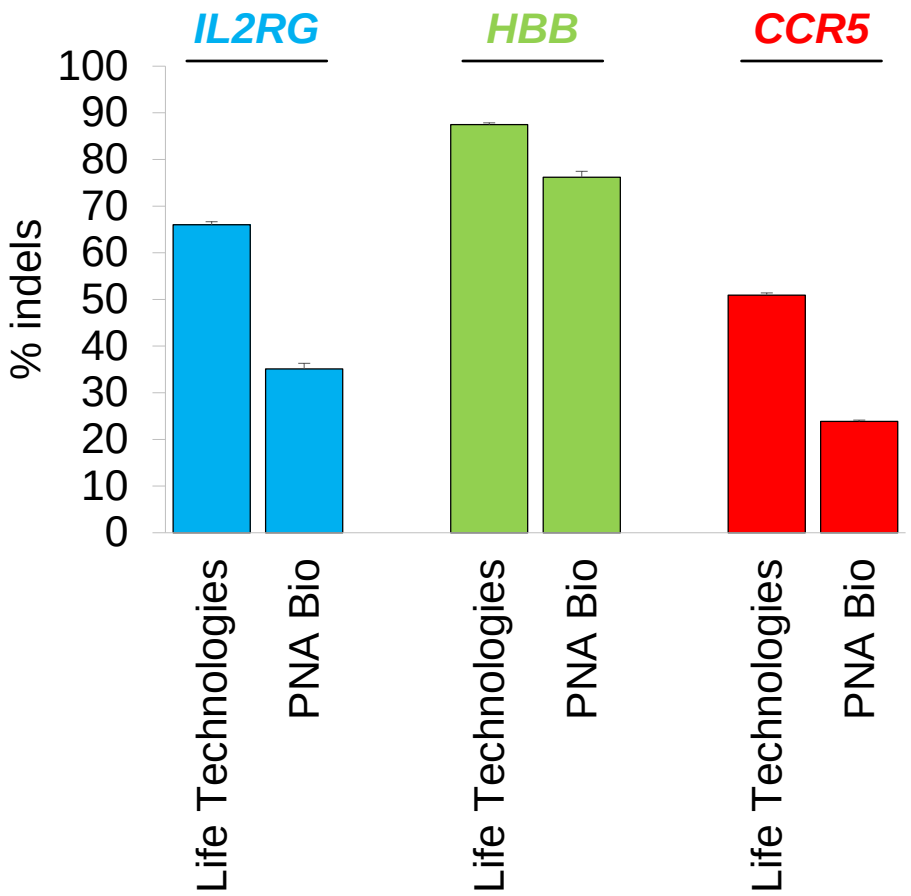
Supplementary Fig. 4. Titration of the MSP sgRNA targeting *IL2RG* and Cas9 mRNA in K562 cells. Measured indel frequencies are averages of three replicates and values are indicated in a heat map. S.e.m. of replicates (n=3) is not indicated for clarity, but all are less than 4% of the measured values shown. Indel frequencies were measured by TIDE analysis of PCR amplicons spanning the sgRNA target site and using a mock-treated sample as a reference control.

Nature Biotechnology: doi:10.1038/nbt.3290



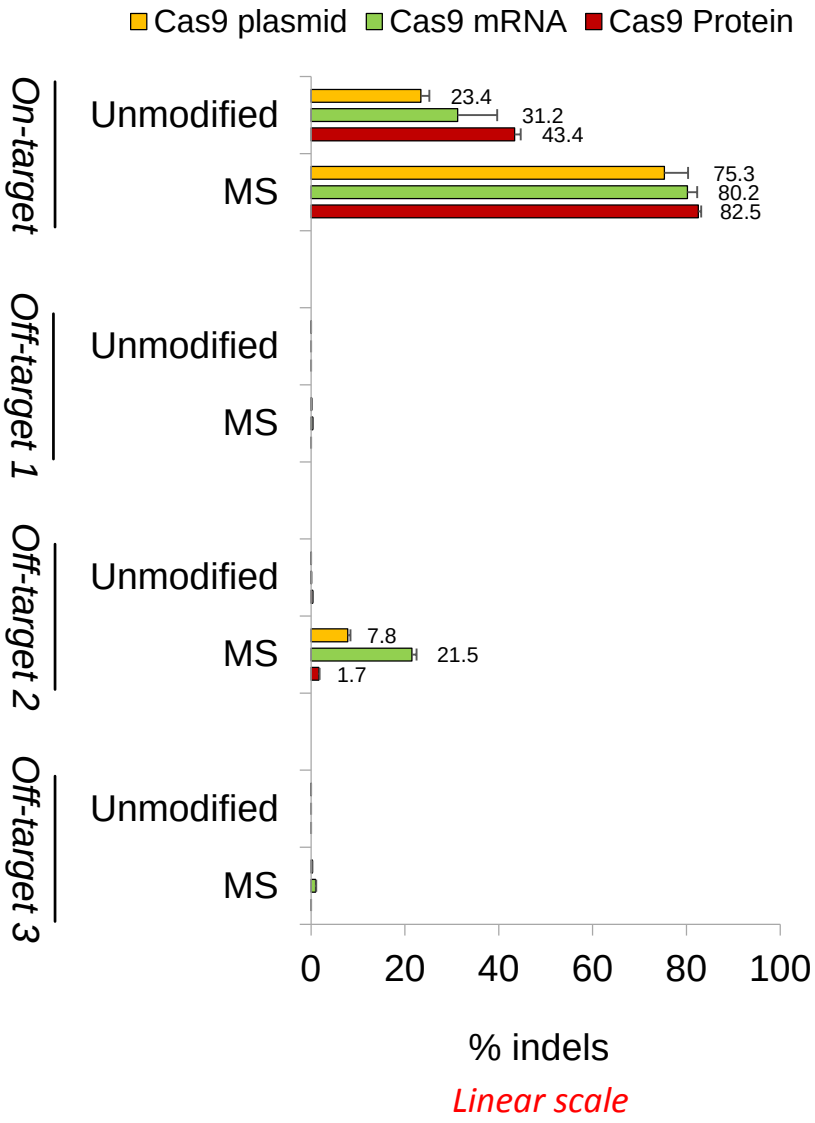
Supplementary Fig. 5. Schematic experimental outline of staggered delivery of sgRNA and Cas9 mRNA. Schematic overview of the experiment yielding data for Fig. 1g. K562 cells were nucleofected at the indicated time points with Cas9 mRNA and/or sgRNAs targeting *IL2RG*. Genomic DNA was extracted 72 hrs after nucleofection of the last component, and indel frequencies were measured by TIDE using a mock-treated sample as a reference control.

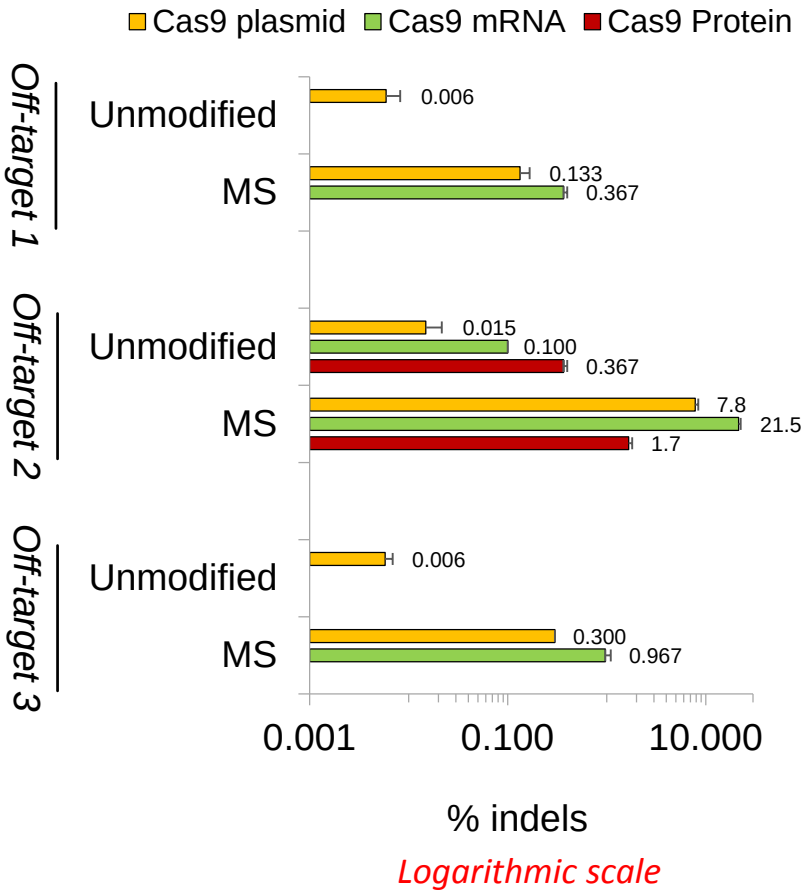
Nature Biotechnology: doi:10.1038/nbt.3290



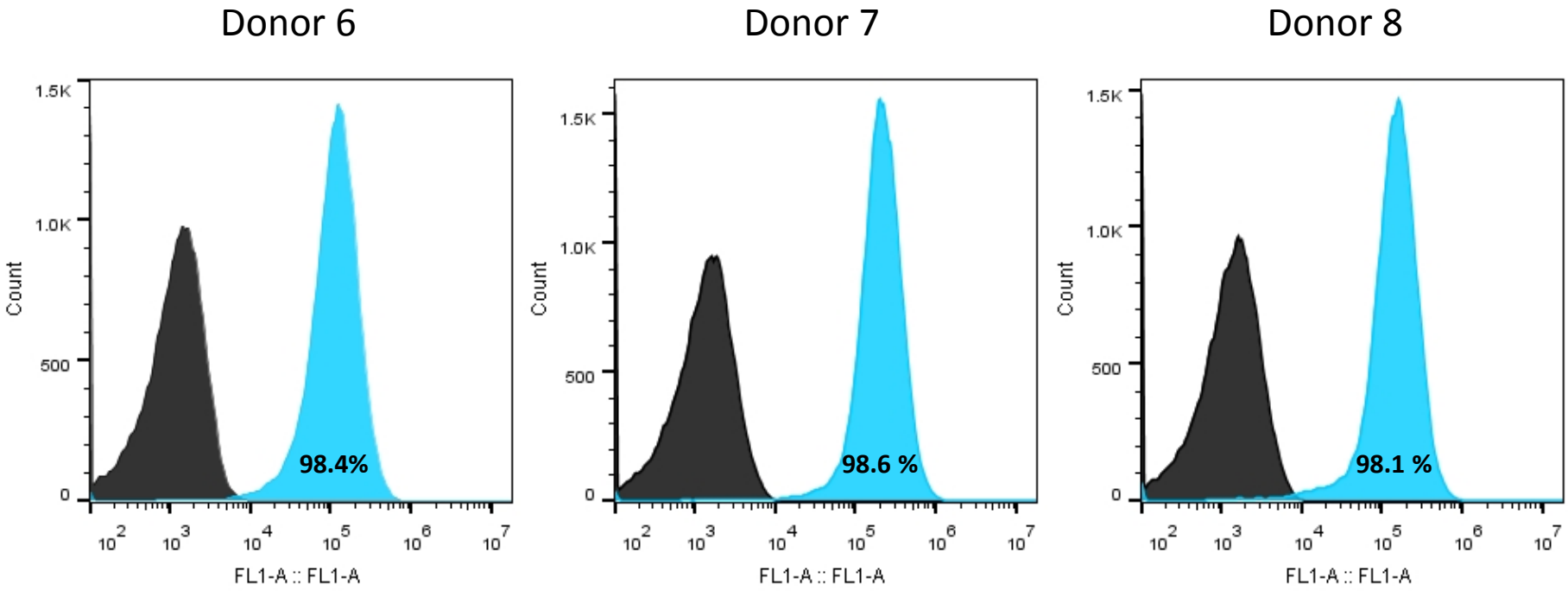
Supplementary Fig. 6. Comparing Cas9 protein from different vendors. Three days after 1 million K562 cells were nucleofected with 15 µg Cas9 protein pre-complexed with a 2.5 molar excess of unmodified sgRNA, genomic DNA was extracted and indel rates were measured by TIDE using a mock-treated sample as a reference control. Bars represent averages + s.e.m., n=3.

Nature Biotechology: doi:10.1038/nbt.3290





Supplementary Fig. 7. Specificity of targeted cleavage mediated by synthetic *IL2RG* sgRNAs and Cas9 plasmid, mRNA, or protein. Target specificity was assessed as in Fig. 1e and Supplementary Fig. 3 using Illumina deep sequencing and displayed on a linear scale (left panel) and logarithmic scale (right panel). 1 million K562 cells were nucleofected with (i) 2 µg Cas9 plasmid + 20 µg sgRNA, (ii) 15 µg Cas9 mRNA + 10 µg sgRNA, or (iii) 15 µg Cas9 protein pre-complexed with 7.6 µg sgRNA (protein:sgRNA molar ratio = 1:2.5). Cas9 plasmid results are the same as shown in Fig. 1e. Bars represent average indel frequencies + s.e.m., n=3.



Supplementary Fig. 8. High RNA nucleofection efficiencies in primary human T cells. Stimulated T cells from three different donors were nucleofected with GFP mRNA three days after stimulation. Expression of GFP was measured three days after nucleofection by flow cytometry. GFP expression in nucleofected cells (blue) is shown relative to mock-transfected cells (black).

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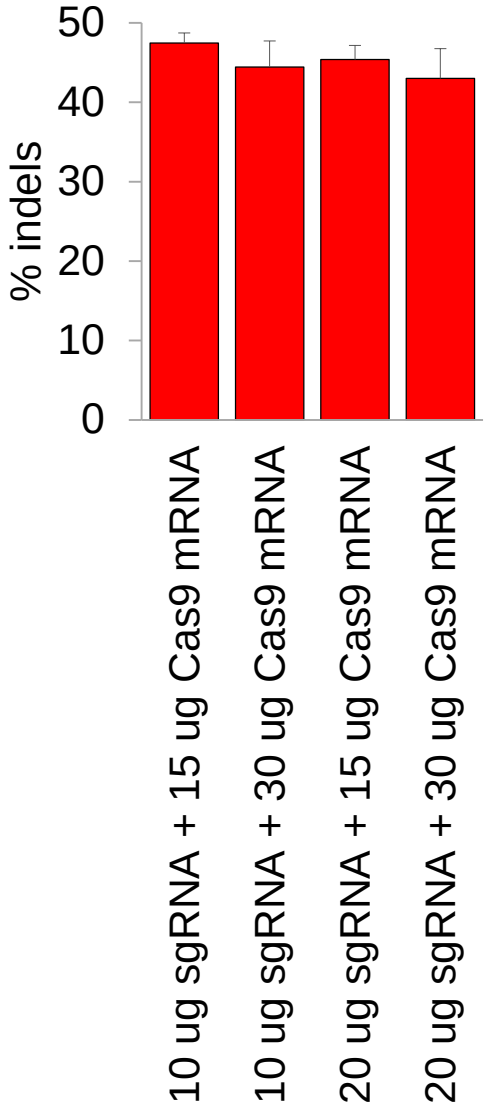
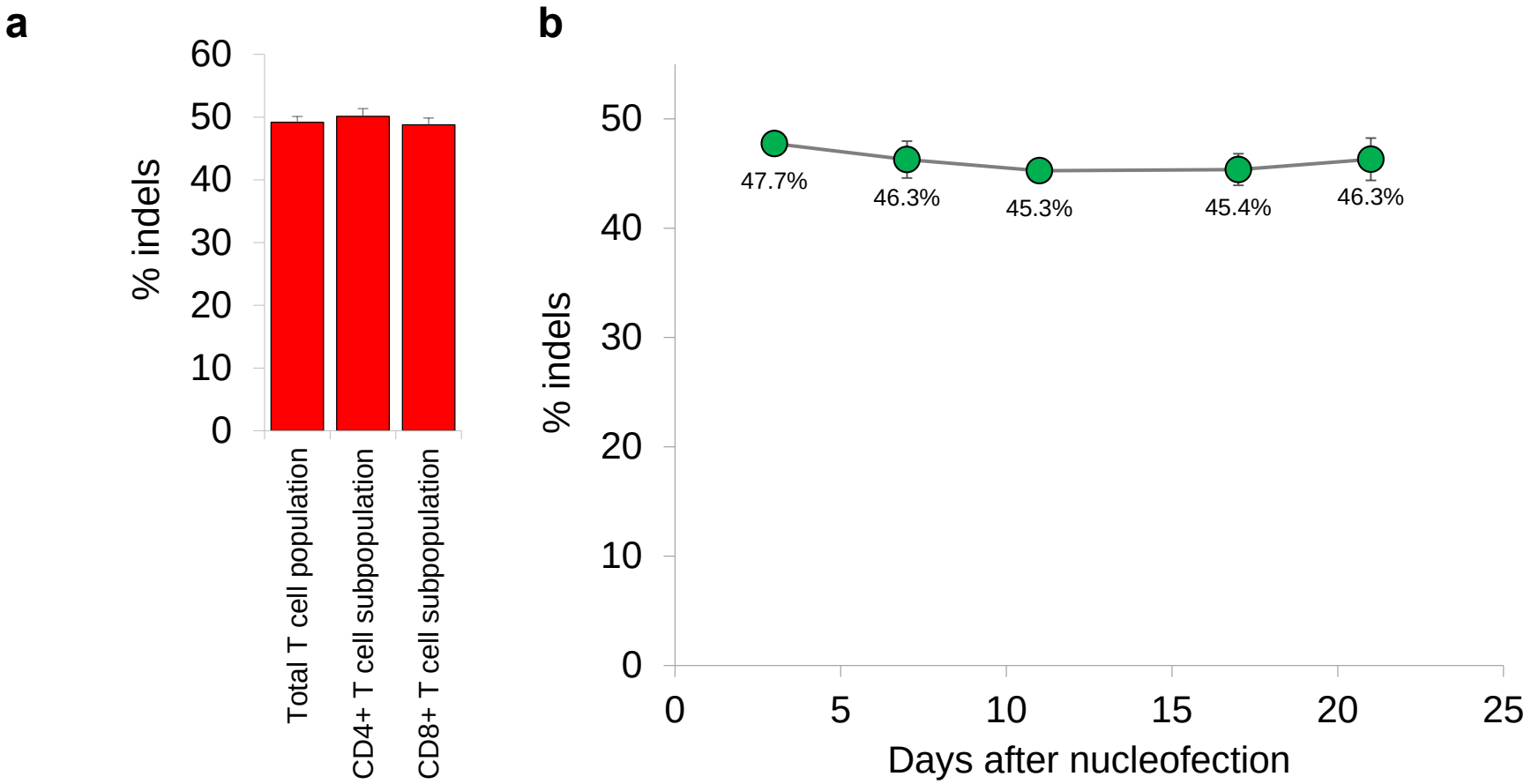


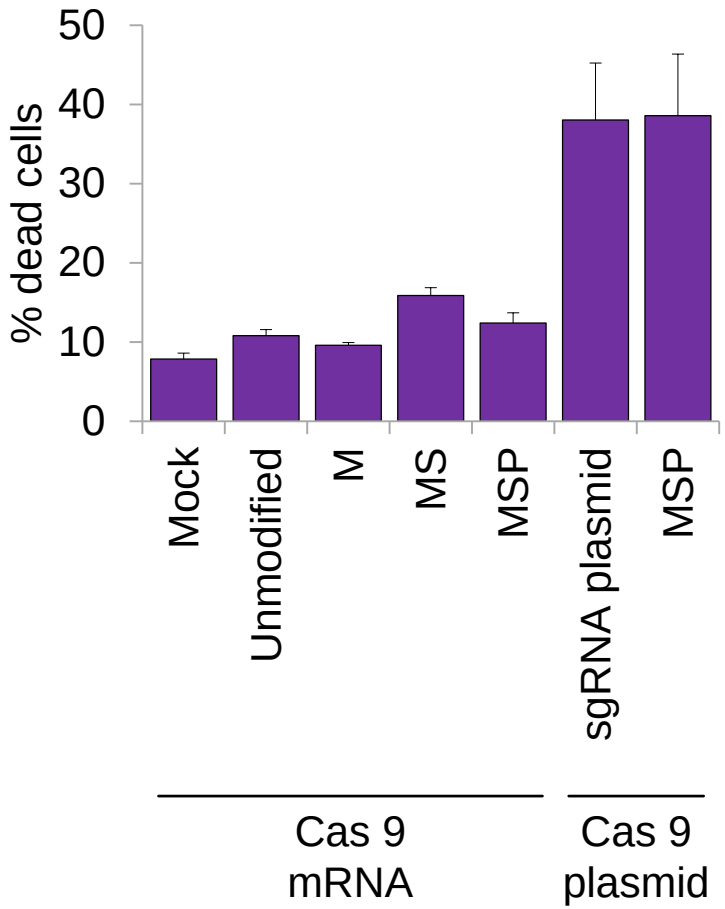
Supplementary Fig. 9. Increasing *CCR5* sgRNA and Cas9 mRNA amounts in T cell nucleofections yield similar indel frequencies. Stimulated T cells were nucleofected with the indicated amounts of the MSP *CCR5* sgRNA and Cas9 mRNA. Indel frequencies were measured by TIDE analysis of PCR amplicons spanning the sgRNA target site and using a mock-treated sample as a reference control. Average indel frequencies are shown for three different T cell donors + s.e.m., n=6.

Nature Biotechnology: doi:10.1038/nbt.3290



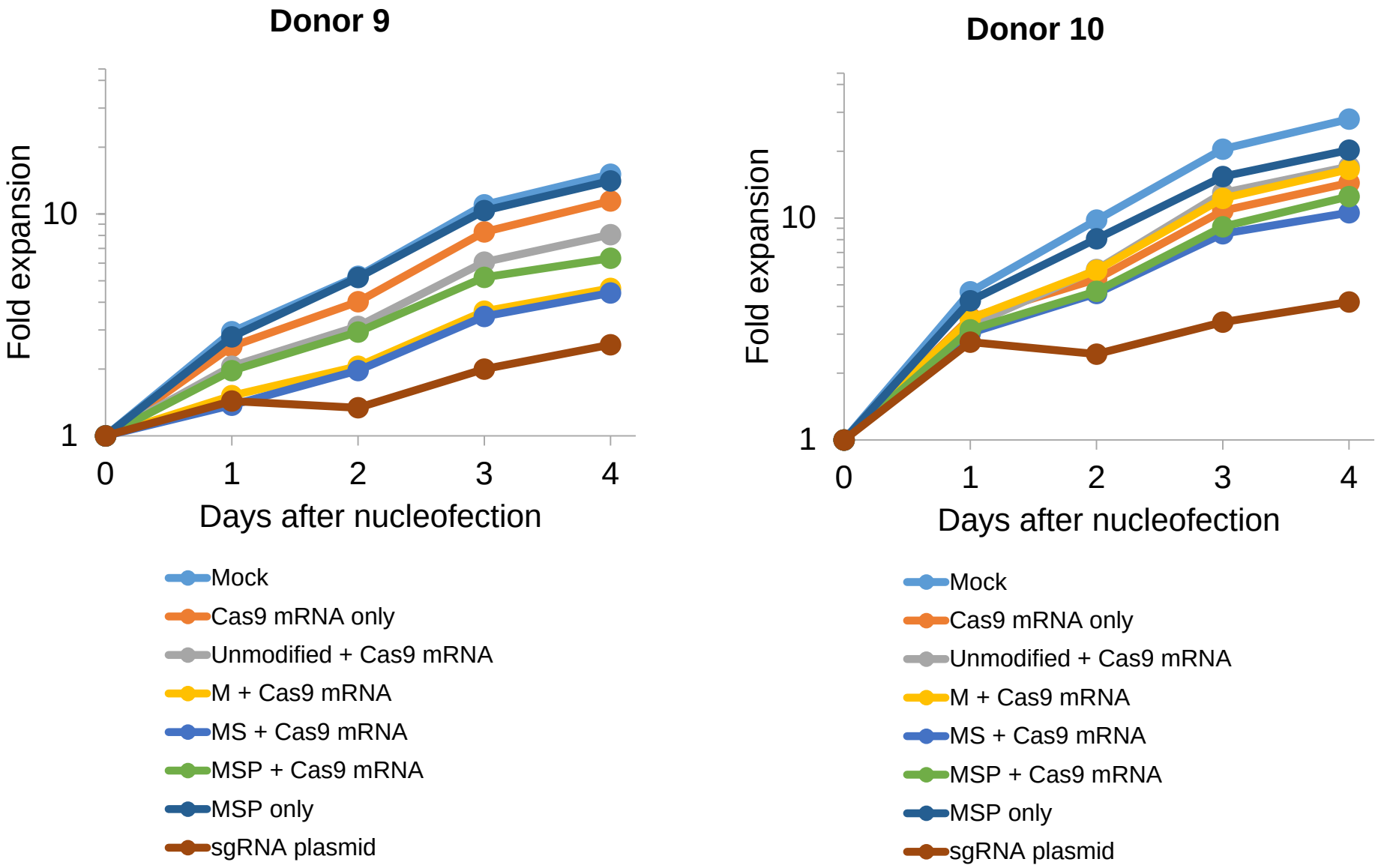
Supplementary Fig. 10. Similar indel frequencies in CD4+, CD8+, and total T cell populations, and stable indel frequencies over time. (a) Stimulated T cells were nucleofected with *CCR5* MSP sgRNA and Cas9 mRNA and subsequently sorted into $CD4^+$ and $CD8^+$ subpopulations. Indel frequencies were measured by TIDE and compared to indel frequencies in the bulk population. Bars represent average indel frequencies for one T cell donor + s.e.m., n=8. **(b)** Stimulated T cells were nucleofected with the *CCR5* MSP sgRNA and Cas9 mRNA. gDNA was extracted from a subset of cells at the indicated time points, and indel frequencies were measured by TIDE analysis of PCR amplicons spanning the sgRNA target site and using a mock-treated sample as reference control. Average indel frequencies are shown for three different T cells donors +/- s.e.m., n=6.

Nature Biotechnology: doi:10.1038/nbt.3290



Supplementary Fig. 11. Lower frequencies of cell death in T cells nucleofected with synthetic sgRNAs and Cas9 mRNA compared to Cas9 plasmid. 1 million stimulated T cells were nucleofected with 10 µg of the indicated synthetic sgRNA and either 15 µg Cas9 mRNA or 1 µg Cas9-encoding plasmid. 1 µg plasmid encoding both the *CCR5* sgRNA and Cas9 protein was included for comparison (sgRNA plasmid). Three days after nucleofection, cells were stained with the LIVE/DEAD cell staining. Bars represent average percentages of dead cells for three different T cell donors + s.e.m., n=6.

Nature Biotechnology: doi:10.1038/nbt.3290



Supplementary Fig. 12. Proliferation assay following nucleofection of synthetic sgRNAs into T cells. Stimulated T cells from two different donors were nucleofected on Day 0, and cell proliferation was monitored using the CellTiter Glo assay. S.e.m. of replicates are not indicated for clarity, but all are less than 15% of the indicated values.

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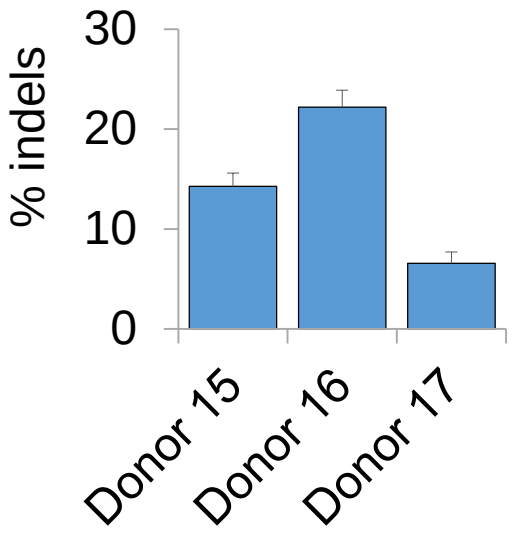
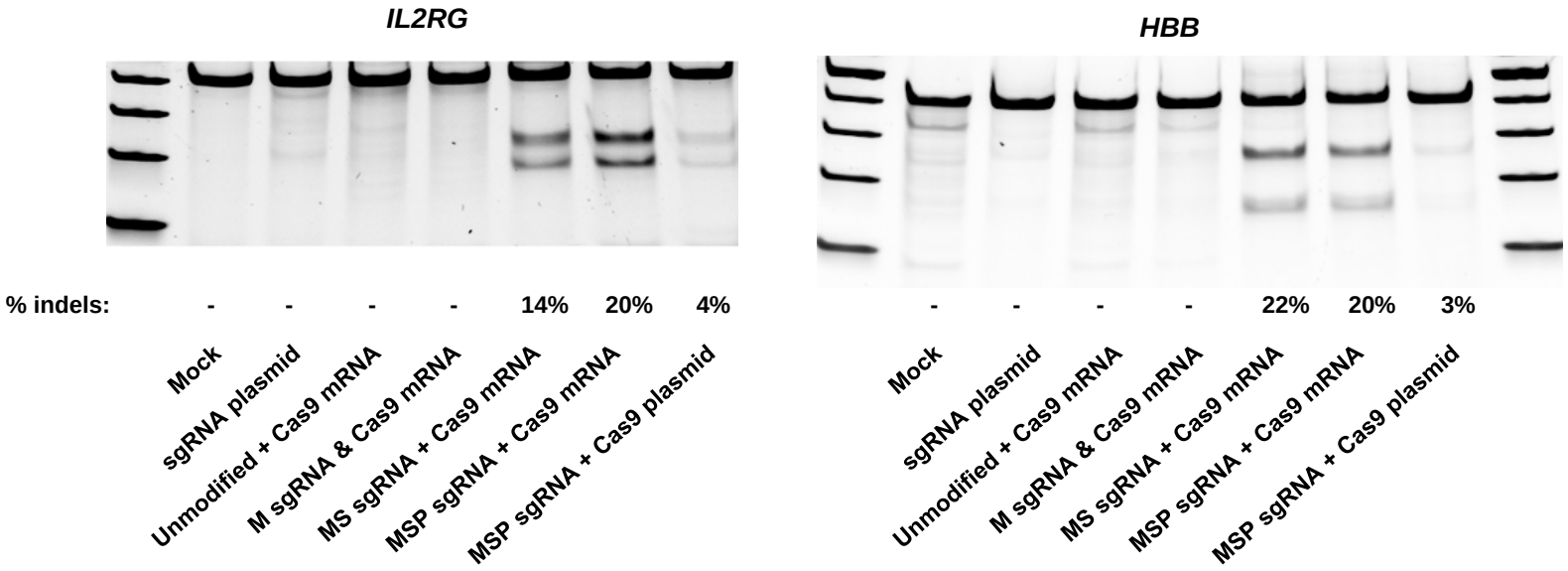


Supplementary Fig. 13. *CCR5* disruption in unstimulated T cells. Unstimulated human T cells from three different donors were nucleofected on the day of isolation with the MS sgRNA and Cas9 mRNA. gDNA was extracted three days after nucleofection and indel frequencies were measured by TIDE using a mock-treated sample as a reference control. Bars represent average + s.e.m., n=2.

Nature Biotechnology: doi:10.1038/nbt.3290



Supplementary Fig. 14. Indel frequencies in mobilized PB CD34$^+$ HSPCs for *IL2RG* and *HBB*. Three days after nucleofection of CD34$^+$ HSPCs, genomic DNA was extracted and indel frequencies were measured by the T7 assay. One representative gel of three biological replicates for each of *IL2RG* and *HBB* is shown.

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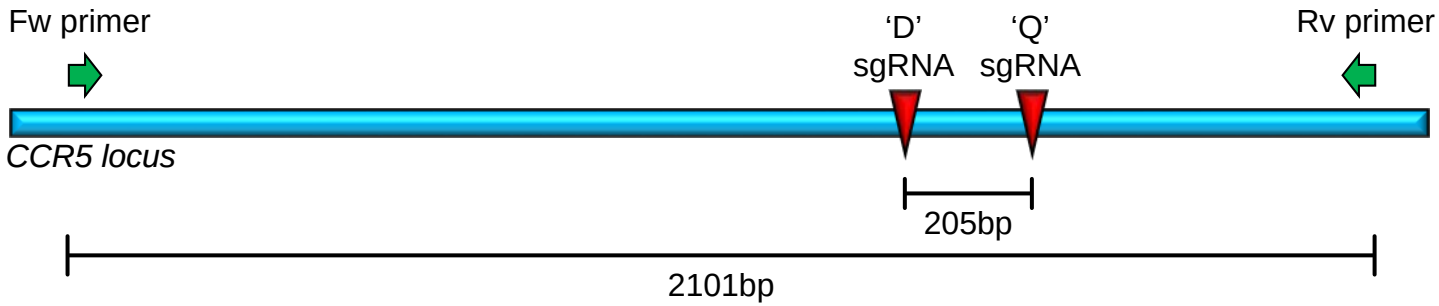
A



B

	T cells		CD34⁺ HSPCs	
	MS	**MSP**	**MS**	**MSP**
Total cloned PCR fragments analyzed	**181**	**186**	**222**	**216**
% allele modification ± s.e.m.	**73.9** (±2.8)	**93.1** (±1.1)	**37.8** (±0.5)	**43.0** (±4.3)
% WT alleles ± s.e.m.	**26.1** (±2.8)	**6.9** (±1.1)	**62.2** (±0.5)	**57.0** (±4.3)
% alleles with deletion between the cut sites ± s.e.m.	**53.4** (±4.2)	**69.7** (±2.1)	**21.1** (±1.8)	**23.1** (±3.1)
% alleles with inverted sequence between the cut sites ± s.e.m.	**5.3** (±2.2)	**9.6** (±2.0)	**5.9** (±2.6)	**6.9** (±2.1)
% indel at both sites ± s.e.m.	**12.7** (±2.4)	**12.6** (±1.6)	**3.6** (±1.2)	**7.9** (±1.9)
% indel at right site only ± s.e.m.	**2.4** (±1.7)	**1.1** (±0.6)	**3.6** (±0.9)	**2.3** (±1.2)
% indel at left site only ± s.e.m.	**0**	**0**	**3.6** (±0.5)	**2.8** (±0.8)

Supplementary Fig. 15. High *CCR5* gene modification frequencies in primary human T cells and CD34⁺ HSPCs using two sgRNAs. Stimulated T cells from three different donors and PB mobilized CD34⁺ HSPCs were nucleofected in triplicate with both the 'D' and 'Q' sgRNA together with Cas9 mRNA. gDNA was extracted three days after nucleofection and the modified region of *CCR5* was PCR-amplified using a pair of primers generating a 2.1kb amplicon for non-modified alleles (panel A). PCR amplicons were subcloned into a plasmid for transformation, and individual colonies representing individual alleles were sequenced, referenced against the expected genomic sequence, and categorized according to the allelic genotype (panel B).

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Supplementary Table 1 Overview of all sgRNAs used in the present study. sgRNA sequences as well as calculated and observed molecular weights are indicated. Nucleotides with 2'-O-methyl modifications are underlined. Modifications in the phosphate backbone are indicated with ● (MS) and ♦ (MSP).

Name	Sequence	Mass Calc. (Da)	Mass Obs. (Da)
HBB unmodified sgRNA	CUUGCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32187.42	32187.84
HBB M sgRNA	CUUGCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32271.54	32271.35
HBB MS sgRNA	C●U●U●GCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU●U●U●U	32367.42	32367.31
HBB MSP sgRNA	C♦U♦U♦GCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU♦U♦U♦U	32619.93	32619.39
IL2RG unmodified sgRNA	UGGUAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32214.40	32214.37
IL2RG M sgRNA	UGGUAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32298.52	32297.01
IL2RG MS sgRNA	U●G●G●UAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU●U●U●U	32394.4	32395.43
IL2RG MSP sgRNA	U♦G♦G♦UAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU♦U♦U♦U	32646.91	32645.39
CCR5 unmodified sgRNA	GGCAGCAUAGUGAGCCCAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32290.51	32289.07
CCR5 M sgRNA	GGCAGCAUAGUGAGCCCAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	32374.63	32375.3
CCR5 MS sgRNA	G●G●C●AGCAUAGUGAGCCCAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU●U●U●U	32470.51	32469.92
CCR5 MSP sgRNA	G♦G♦C♦AGCAUAGUGAGCCCAGAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU♦U♦U♦U	32723.02	32721.96
CCR5 'D' MS sgRNA	U●C●A●CUAUGCUGCCGCCCAGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU●U●U●U	32,281.32	32,282.52
CCR5 'D' MSP sgRNA	U♦C♦A♦CUAUGCUGCCGCCCAGUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU♦U♦U♦U	32,533.83	32,533.55
CCR5 'Q' MS sgRNA	G●C●U●GUGUUUGCGUCUCUCCCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU●U●U●U	32,252.22	32,253.21
CCR5 'Q' MSP sgRNA	G♦C♦U♦GUGUUUGCGUCUCUCCCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU-AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU♦U♦U♦U	32,504.73	32,504.63

Supplementary Table 2

Tables showing the numbers on which Figs. 1c, 1e, and Supplementary Fig. 3 are based. Specificity of targeted cleavage mediated by synthetic sgRNAs as performed in Fig. 1c with 2 µg Cas9 plasmid and either 1 µg sgRNA (upper panel) or 20 µg sgRNA (lower panel). Indel frequencies were measured by deep sequencing of PCR amplicons from the targeted genomic loci and three bioinformatically predicted off-target loci for each gene. Average values are shown +/- s.e.m., n=3.

2 µg Cas9 plasmid and 1 µg sgRNA

		MOCK	UNMODIFIED	M	MS	MSP
IL2RG	**On-target**	0.16 ±0.03	2.43 ±0.23	13.47 ±0.54	68 ±1.06	75.73 ±1.30
	Off-target 1	0.00	0.00	0.00	0.00	0.00
	Off-target 2	0.00	0.00	0.10 ±0.00	1.00 ±0.10	0.37 ±0.03
	Off-target 3	0.00	0.00	0.00	0.00	0.00
HBB	**On-target**	0.15 ±0.05	1.3 ±0.08	12.4 ±0.28	48.17 ±0.39	38.68 ±0.85
	Off-target 1	0.01 ±0.01	0.84 ±0.07	7.01 ±0.36	27.31 ±0.60	9.51 ±0.25
	Off-target 2	0.00	0.00	0.01 ±0.00	0.00	0.00
	Off-target 3	0.00	0.00	0.00	0.00	0.00
CCR5	**On-target**	0.02 ±0.01	4.26 ±0.18	3.41 ±0.23	24.6 ±1.25	22.78 ±1.76
	Off-target 1	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.02 ±0.00
	Off-target 2	0.01 ±0.01	0.01 ±0.00	0.01 ±0.00	0.08 ±0.03	0.54 ±0.10
	Off-target 3	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00

2 µg Cas9 plasmid and 20 µg sgRNA

		UNMODIFIED	M	MS	MSP	sgRNA PLASMID
IL2RG	**On-target**	23.4 ±1.8	48.13 ±0.4	75.3 ±5.1	83.27 ±0.7	70.53 ±0.01
	Off-target 1	0.00	0.03 ±0.03	0.13 ±0.03	0.00	0.00
	Off-target 2	0.13 ±0.03	1.20 ±0.06	7.83 ±0.58	2.77 ±0.24	0.07 ±0.00
	Off-target 3	0.00	0.10 ±0.00	0.30 ±0.00	0.10 ±0.00	0.17 ±0.00
HBB	**On-target**	19.42 ±0.27	40.99 ±1.4	65.91 ±0.62	60.71 ±0.25	31.11 ±0.04
	Off-target 1	9.2 ±0.38	33.56 ±2.3	55.1 ±0.97	19.08 ±0.49	25.44 ±0.04
	Off-target 2	0.00	0.00	0.00	0.00	0.00
	Off-target 3	0.00	0.00	0.00	0.00	0.00
CCR5	**On-target**	11.04 ±2.5	14.87 ±2.1	56.3 ±3.3	52.19 ±4.5	45.86 ±0.03
	Off-target 1	0.02 ±0.01	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00
	Off-target 2	0.01 ±0.00	0.02 ±0.00	1.75 ±0.18	5.32 ±0.68	0.02 ±0.00
	Off-target 3	0.01 ±0.00	0.01 ±0.00	0.02 ±0.01	0.02 ±0.00	0.01 ±0.00

Supplementary Table 3 List of on- and off-target loci interrogated by deep sequencing of PCR amplicons. For the *CCR5*, *HBB*, and *IL2RG*-targeting CRISPR experiments, the intended genomic target sequence ('ON') and three computationally predicted OFF-target sequences ('OFF1-3') are presented with their genomic location (human genome build assembly hg38). Chosen off-target sequences were predicted top-scorers by both the COSMID[1] and Optimized CRISPR Design[2] webtools (MIT design), except HBB-OFF3 which was only predicted to have significant activity by COSMID. Predicted target activity increases with increasing COSMID score values and decreasing 'MIT design' score values. The PAM sites and mismatches in off-target sequences are indicated by red colored and bolded text, respectively.

Target ID	Target site sequence (5'→ 3')	Genomic location	Strand	Target score	
				COSMID[1]	MIT design[2]
CCR5 targeting guide RNA					
ON	GGCAGCATAGTGAGCCCAGA**AGG**	Chr3:46373153-46373175	-	0	56
OFF1	ATCATCATAGTGAGCCCAGA**GAG**	Chr3:15440658-15440680	+	0.44	2.4
OFF2	ACCAGCAGAGTGAGCCCAGA**GGG**	Chr4:3744369-3744391	+	0.52	2.6
OFF3	AGGAGCAGAGTGAGCCCAGA**GAG**	Chr15:92469456-92469478	+	0.54	2.6
HBB targeting guide RNA					
ON	CTTGCCCCACAGGGCAGTAA**CGG**	Chr11:5226968-5226990	+	0	65
OFF1	TCAGCCCCACAGGGCAGTAA**GGG**	Chr9:101833584-101833606	+	0.4	2.3
OFF2	CCTCTCCCACAGGGCAGTAA**AGG**	Chr17:68628098-68628120	-	0.49	2.4
OFF3	TTTTCCCCAAAGGGCAGTAA**TAG**	Chr13:109165988-109166010	+	0.79	N/A
IL2RG targeting guide RNA					
ON	TGGTAATGATGGCTTCAACA**TGG**	ChrX:71111519-71111541	+	0	49
OFF1	TGGGAAGGATGGCTTCAACA**CAG**	Chr7:151485304-151485326	-	0.4	3.9
OFF2	TGGTGAGGATGGCTTCAACA**CGG**	Chr1:167730172-167730194	-	0.42	3.7
OFF3	TGGTAATGATGACTTCAACA**TAG**	Chr3:72764801-72764823	-	0.8	49.2

Supplementary Table 4 List of oligonucleotide primers used for generation of on- and off-target amplicons to quantify indel rates by deep sequencing. The gene-specific hybridization sequences of the gene-specific amplicon primers and barcodes of the illumina barcoding primers are indicated with underlined and bolded text, respectively.

Primer name	Primer sequence (5'→ 3')
Gene-specific amplicon primers	
CCR5_ON-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCAAACACAGCATGGACGACA
CCR5_ON-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTGAAGAGCATGACTGACA
CCR5_OFF1-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGGGGAAGCAGTCTGGACTTAGA
CCR5_OFF1-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTGCCATTAAATCCACCAAA
CCR5_OFF2-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCAGCGAGTCGAGTTCAGGTG
CCR5_OFF2-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGCTACCTACCCCAGGTTCT
CCR5_OFF3-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCTCACCAACACTGCCGAAT
CCR5_OFF3-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGCATATAGTGCTCCCCACT
HBB_ON-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCTGTCTCCACATGCCCAGT
HBB_ON-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAGGGCAGAGCCATCTATTG
HBB_OFF1-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCCCGTTCTCCACCCAATAG
HBB_OFF1-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGATTTCCAGGCTATGCTTCCA
HBB_OFF2-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCGTTGGCAGGGAGACTTACCA
HBB_OFF2-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCATGGTACGACTGTTCTCA
HBB_OFF3-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTGGGGCCTTCAAGTGTTCTT
HBB_OFF3-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTGTGCTCCTATGCCTGGTT
IL2RG_ON-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCATTGGGCGTCAGAATTGTC
IL2RG_ON-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGTTCTTTCCACCGGAAGC
IL2RG_OFF1-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCTCCGGAAGTTATTCAAGTCTGA
IL2RG_OFF1-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTCTGGCATCAGAGCACAAA
IL2RG_OFF2-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCCTGGGCCATATCAAGAGAC
IL2RG_OFF2-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTTTGGGGTGATGTTTGTG
IL2RG_OFF3-fwd	CGACAGGTTCAGAGTTCTACAGTCCGACGATCCACAACAGTTGACCCAGGAA
IL2RG_OFF3-rev	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCAACCCAGGTCTCTGAAC
Illumina barcoding primers	
P5-BC_A-fwd	AATGATACGGCGACCACCGAGATCTACAC**TAGAGCTC**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC_B-fwd	AATGATACGGCGACCACCGAGATCTACAC**TCATAGCG**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC_C-fwd	AATGATACGGCGACCACCGAGATCTACAC**GTAGCACT**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC_D-fwd	AATGATACGGCGACCACCGAGATCTACAC**GCTCATAG**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC_E-fwd	AATGATACGGCGACCACCGAGATCTACAC**ATGCATCG**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC_F-fwd	AATGATACGGCGACCACCGAGATCTACAC**AGTCGATC**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC-G_fwd	AATGATACGGCGACCACCGAGATCTACAC**CACTGTGA**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P5-BC-H_fwd	AATGATACGGCGACCACCGAGATCTACAC**CGAGTATC**CGACAGGTTCAGAGTTC-TACAGTCCGACGATC
P7-BC-A_rev	CAAGCAGAAGACGGCATACGAGAT**TAGAGCTC**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT

P7-BC_B rev	CAAGCAGAAGACGGCATACGAGAT**TCATAGCG**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC_C rev	CAAGCAGAAGACGGCATACGAGAT**GTAGCACT**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC_D rev	CAAGCAGAAGACGGCATACGAGAT**GCTCATAG**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC_E_rev	CAAGCAGAAGACGGCATACGAGAT**ATGCATCG**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC_F_rev	CAAGCAGAAGACGGCATACGAGAT**AGTCGATC**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC-G_ rev	CAAGCAGAAGACGGCATACGAGAT**CACTGTGA**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT
P7-BC-H_rev	CAAGCAGAAGACGGCATACGAGAT**CGAGTATC**GTGACTGGAGTTCAGAC-GTGTGCTCTTCCGATCT

Supplementary Table 5 List of *CCR5*, *HBB*, and *IL2RG* on- and off-target amplicons generated for deep sequencing analysis of indel rates. Amplicon sizes (of unedited genomic DNA) range from 183-220 bp, with a minimum of 50 bp from the target site to the hybridization sequence of the gene-specific primer. The hybridization sequences used for amplicon generation from genomic DNA and putative CRISPR-target sites are indicated in underlined and bolded text, respectively.

Target ID	Target amplicon sequence (5'→ 3')
CCR5 targeting guide RNA	
ON	CAAACACAGCATGGACGACAGCCAGGTACCTATCGATTGTCAGGAGGATGATGAAGAA-GATTCCAGAGAAGAAGCCTATAAAATAGAGCCCTGTCAAGAGTTGACACATT-GTATTTCCAAAGTCCCACTGGGC**GGCAGCATAGTGAGCCCAGAAGG**GGACAGTAA-GAAGGAAAAACAGGTCAGAGATGGCCAGGTTGAGCAGGTAGATGTCAGTCATGCTCTTCAGCC
OFF1	GGGGAAGCAGTCTGGACTTAGAAAGGAAATAGGTGGTCTGTCATAGGGGCTTTCATT-AGAGTTAAACTTCATAGAGTCAACTGTTTC**ATCATCATAGTGAGCCCAGAGAG**CCAC-TGCCCAGCAGCATGCTCACACCACCTACCCTAGTGTAGGTAATAGGTCTACGCTAG-GACCCCGTGCTGGGCTCTCAGCCCATCATGAGATTTTGGTGGATTTAATGGCAGG
OFF2	AGCGAGTCGAGTTCAGGTGGGAGCAGAGGGCGCCCACCAGCAGAGCGAGTCGAG-TCCAGGCGGGAGCAGAGGGCGCAC**ACCAGCAGAGTGAGCCCAGAGGG**TTTAAA-GAAGGGGCGGTCTCTACGGTATGGGTAGAGTCAGGGGAACTAGGAAAGGACAGAGCAGAAC-CTGGGGTAGGTAGCC
OFF3	TCTCACCAACACTGCCGAATGTCATCTCTTCTCATCTTTATCTCTATTCTTT-GCTTCCTGTCTTCAGGGCTCTTCCCTTGGCATTCACC**AGGAGCAGAGTGAGCCCAGA-GAG**CTGAGTGGTATCCCTTCTTCTTGGGTCCCTGAGCCCTGACCTGGAGCAATGCTGTGAGA-CAGCAGGAAAGGAGGGGAGTGTGGAGTGGGGAGCACTATATGCCA
HBB targeting guide RNA	
ON	TCTGTCTCCACATGCCCAGTTTCTATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATAC-CAACCTGCCCAGGGCCTCACCACCAACTTCATCCACGTTCAC**CTTGCCCCACAGGGCAG-TAACGG**CAGACTTCTCCTCAGGAGTCAGATGCACCATGGTGTCTGTTTGAGGTTGCTAG-TGAACACAGTTGTGTCAGAAGCAAATGTAAGCAATAGATGGCTCTGCCCTG
OFF1	TCCCGTTCTCCACCCAATAGCTATGGAAAGGGGAAGATCCCAGAGAACTTGGA-TAGGAAAGGTGAAGTCAGAGCAGTGCT**TCAGCCCCACAGGGCAGTAAGGG-**CAGCCTTCCTCTAAATACCAGATTCCCAAATCTGGCTGTGCTTTCAATTTGGGAGTT-GGACATACTGCTAAACTATAATTTCTTAGGCCGTACCTAAAATATATTATGGAA-
OFF2	GTTGGCAGGGAGACTTACCAGCTTCCCGTATCTCCCTCCACATGGAGGCAGGACAC-GCTCTGGCCTTGCCCACCCTCCCACTAG**CCTCTCCCACAGGGCAGTAAAGG**TGAGTCTGGGA-GAAAGAACCGGTCAGACTTAGTTCAGCTCCACCCTTTCCTCCTGGGAGTGAG-TCTTTCCAAGACAGAGCATGTTTTTTTCTACCCCTCAGTGAGAACAGTCGTACCATGGG
OFF3	TGGGGCCTTCAAGTGTTCTTCCCAAGAGTCAGAGTGAACCAGAACCAAGAACCATGTT-GAGTTGCCCAGATGTAACCAGGCCTACAGGTACCTGGGAGAAACACGTG-TACA**TTTTCCCCAAAGGGCAGTAATAG**CATCCTAGGCTTCAAAACATTCATAGAAAC-CATTTTTCAAATGCAAAGTCCAACACAGTTAGAAATAACCAGGCATAGGAGCACAC
IL2RG targeting guide RNA	
ON	CATTGGGCGTCAGAATTGTCGTGTTCAGCCCCACTCCCAGCAGGGGCAGCTGCAGGAA-TAAGAGGGATGTGAA**TGGTAATGATGGCTTCAACATGG**CGCTTGCTCTTCATTCCCTGGGTG-TAGTCTGTCTGTGTCAGGAACCTGGGTCCCTCACCCACTACCCCTCCCCACCCACAC-GTTTCCTCTGTCATAGCTTCCGGTGGAAAGAACCT
OFF1	TCCGGAAGTTATTCAAGTCTGATTTTCTTTCCTCCCTTGTCAGGGAAAAGAAGTT-GTGACAAATTGCTTGGATCCTTAAGCTTAAG**TGG-GAAGGATGGCTTCAACACAG**AACATCTGTTTCATTGCTGTTTTATCCGTCAG-TAAAACTGTTACTTCTTTTATGTACTAAAAGTTCTTAGCACTTAACTAATATTAGCTCTTT-

OFF2	CCTGGGCCATATCAAGAGACTCTGCCTCAAAAAAGAAAAGAAAGAAAGAAAAA- GAAAAAAAAAAGAACATCATTAAAAATCCCTGAGGAGCATTTAGAGTATTGGGTGG- CACAAACAGATTCTGCATGAT**TGGTGAGGATGGCTTCAACACGG-** CAGCTTTATTCCTCTTTAACAGAGTCAGCAGCATCAAGGCATGAGGGATCTT-
OFF3	CACAACAGTTGACCCAGGAACAGGGGGAACCTCCCACCATTCCCATCCCACTGTTT- GATCAGATCCAAGAATCCACAATATTGAGAGTGAATGAAAAGTGTCAGC**TGG-** **TAATGATGACTTCAACATAG**TCAGAACTCTTTGGGGTGTTCCAAACATCATGGTGCAT- ATGTATTACCTGGGAGTCTTGTTAAAAAGACTCCTGTTCAGAGACCTGGGTTGGG

References

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2. Hsu, P.D. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature biotechnology* **31**, 827-832 (2013).

Supplementary Note 1

IL2RG targeting vector sequence

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IL2RG homology arms

Chimeric intron

GFP

BGH Poly(A)

HBB targeting vector sequence

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gttccgcgcacatttccccgaaaagtgccac

HBB homology arms

EF1α promoter

GFP

BGH Poly(A)

Nature Biotechnology: doi:10.1038/nbt.3290

CCR5 targeting vector sequence

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HBB homology arms

EF1α promoter

GFP

WPRE

BGH Poly(A)

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Supplementary Note 2

Generation of CRISPR on- and predicted off-target amplicons for deep sequencing

For each targeted gene locus, 10^6 K562 cells were transfected with either 1 µg or 20 µg of the synthetic guide RNAs (unmodified, M, MS or MSP) and 2 µg of the Cas9 expression plasmid (PX330), 2 µg of sgRNA plasmid encoding both the sgRNA and Cas9 protein (positive control), or 2 µg of PX330 only (Cas9 only, negative control). Additionally, for experiments targeting the *IL2RG* gene locus, 10^6 K562 cells were transfected with 15 µg Cas9 mRNA and 10 µg of synthetic guide RNA (unmodified or MS), 15 µg Cas9 protein pre-complexed with 7.6 µg synthetic guide RNA (unmodified or MS). Genomic DNA from these samples and mock transfection samples (2^{nd} negative control) was extracted 72 hours post transfection using QuickExtract™ DNA Extraction Solution (Epicentre, Madison, WI) according to the manufacturer's specifications. 40ng of genomic DNA was used as a template for PCR amplification of the on-target and three computationally predicted off-target loci (see **Supplementary Table 3**), using PfuUltra II HS 2x Master Mix (Agilent Technologies, Santa Clara, CA) and gene-specific primers that tag the amplicon ends with sequencing primers utilized in deep sequencing by the MiSeq (Illumina, San Diego, CA) platform (see **Supplementary Table 4**). A second PCR reaction was carried out on the on- and off-target amplicons (see **Supplementary Table 5**) to append additional Illumina sequencing adaptors (i.e. P5, P7) and custom, dual 8-bp barcodes, uniquely identifying the corresponding transfection treatments. Following the 2^{nd} PCR, barcoded amplicons were quantified by Agilent D1000 TapeStation, pooled in equimolar concentrations before purification with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufac-turer's instructions. The purified library was sequenced on an Illumina MiSeq DNA sequencer, 2 x 201 cycles with dual indexing by a NGS DNA sequencing service (Seqmatic, Fremont, CA).

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