OMTN, Volume *24*

Supplemental information

Efficient correction of Duchenne muscular dystrophy mutations by SpCas9 and dual gRNAs

Xi Xiang, Xiaoying Zhao, Xiaoguang Pan, Zhanying Dong, Jiaying Yu, Siyuan Li, Xue Liang, Peng Han, Kunli Qu, Jonas Brorson Jensen, Jean Farup, Fei Wang, Trine Skov Petersen, Lars Bolund, Huajing Teng, Lin Lin, and Yonglun Luo

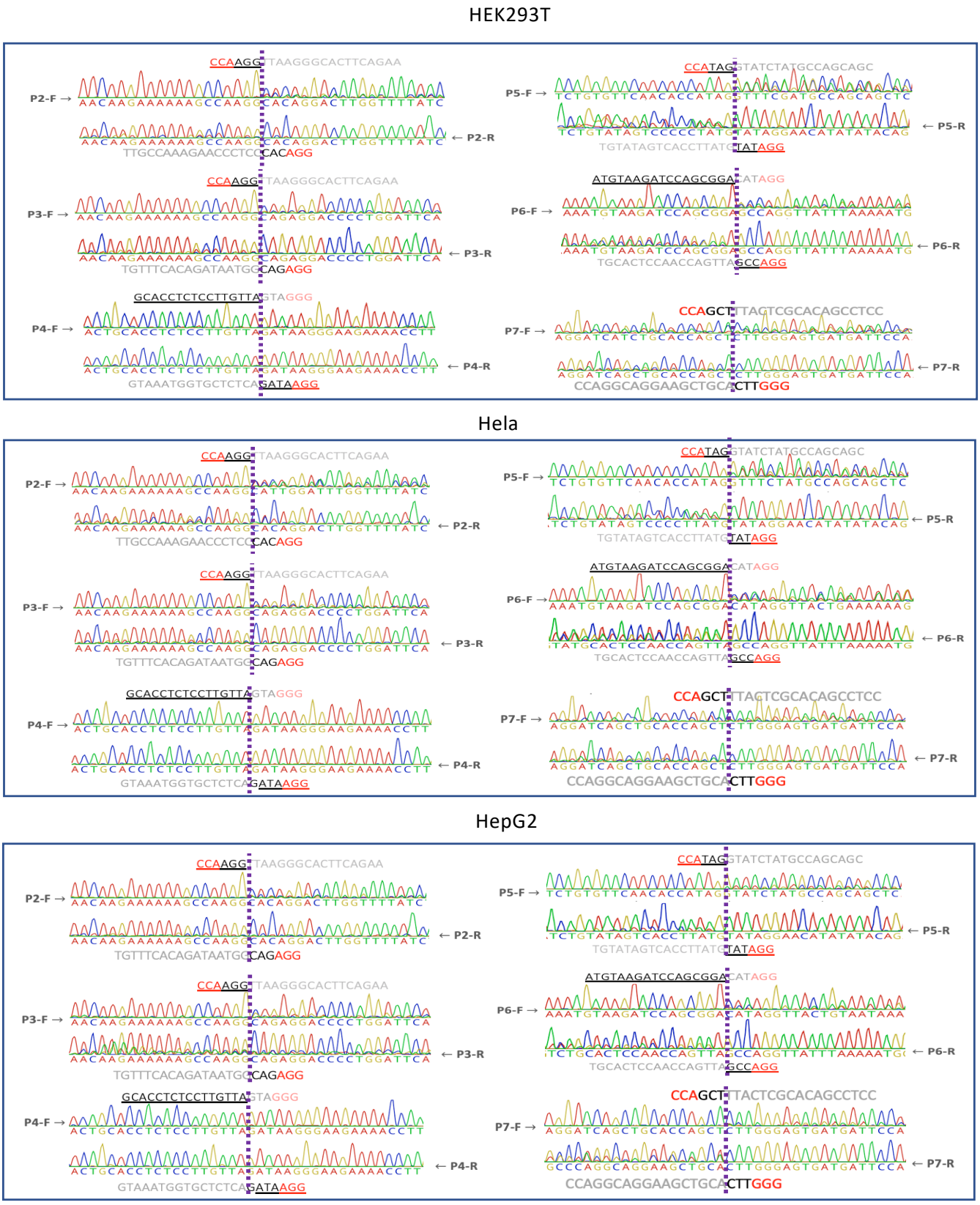
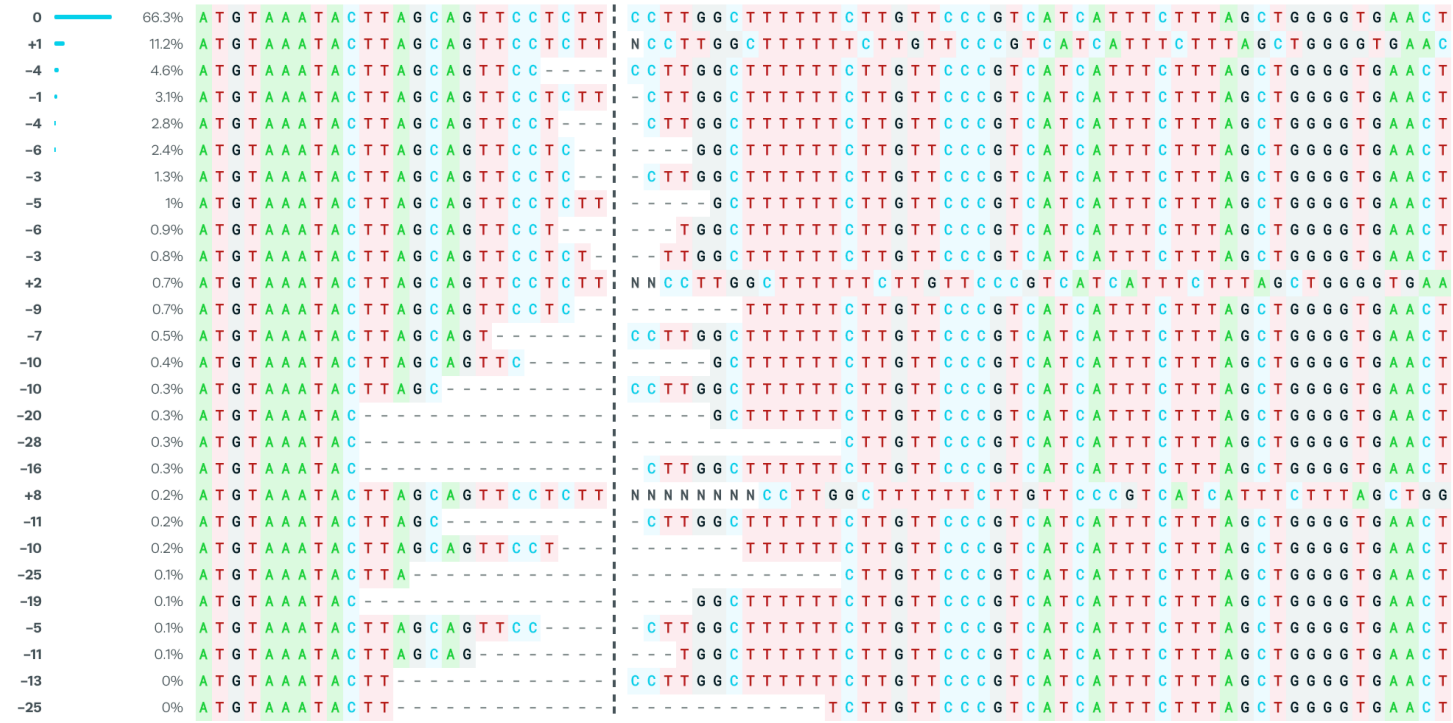


Figure S1.
Sanger sequencing results of the additional 6 loci after pair-gRNAs cleavage in three human cell lines.

HEK293T

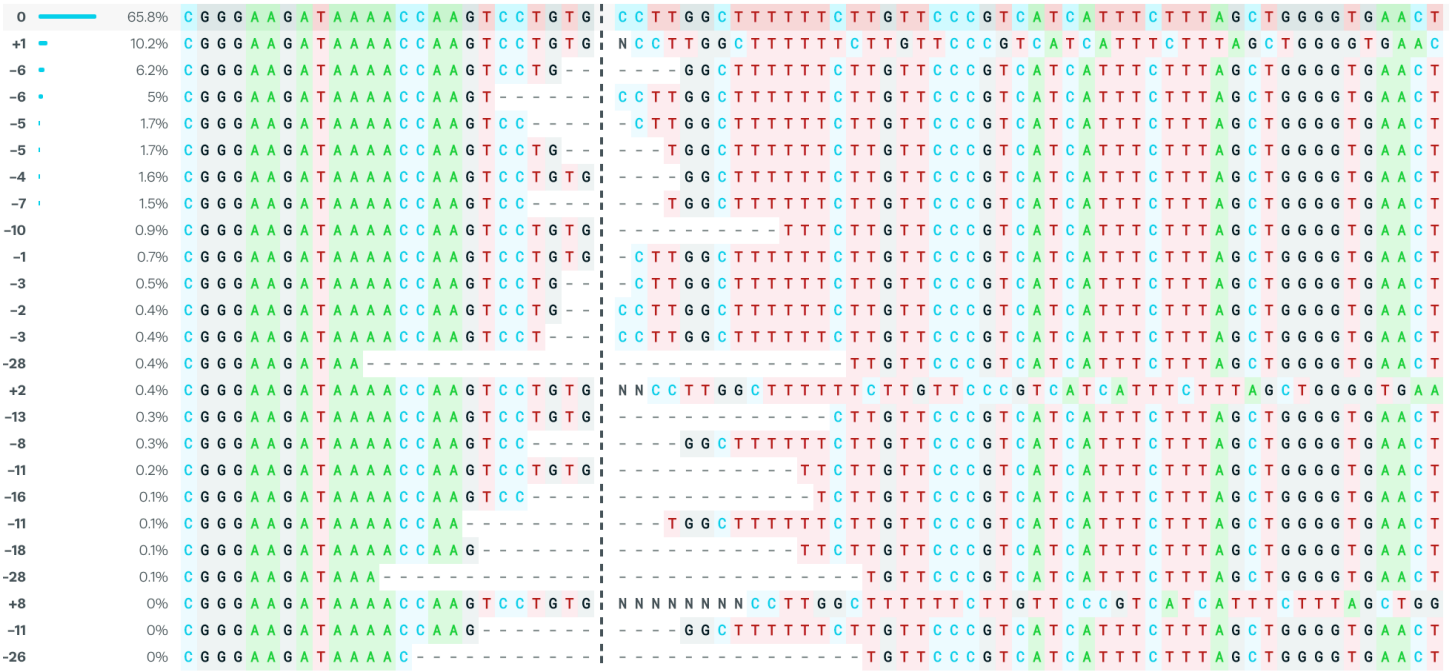
P1 67.0%

CAAGAAAAAAGCCAAGGAAGAGG



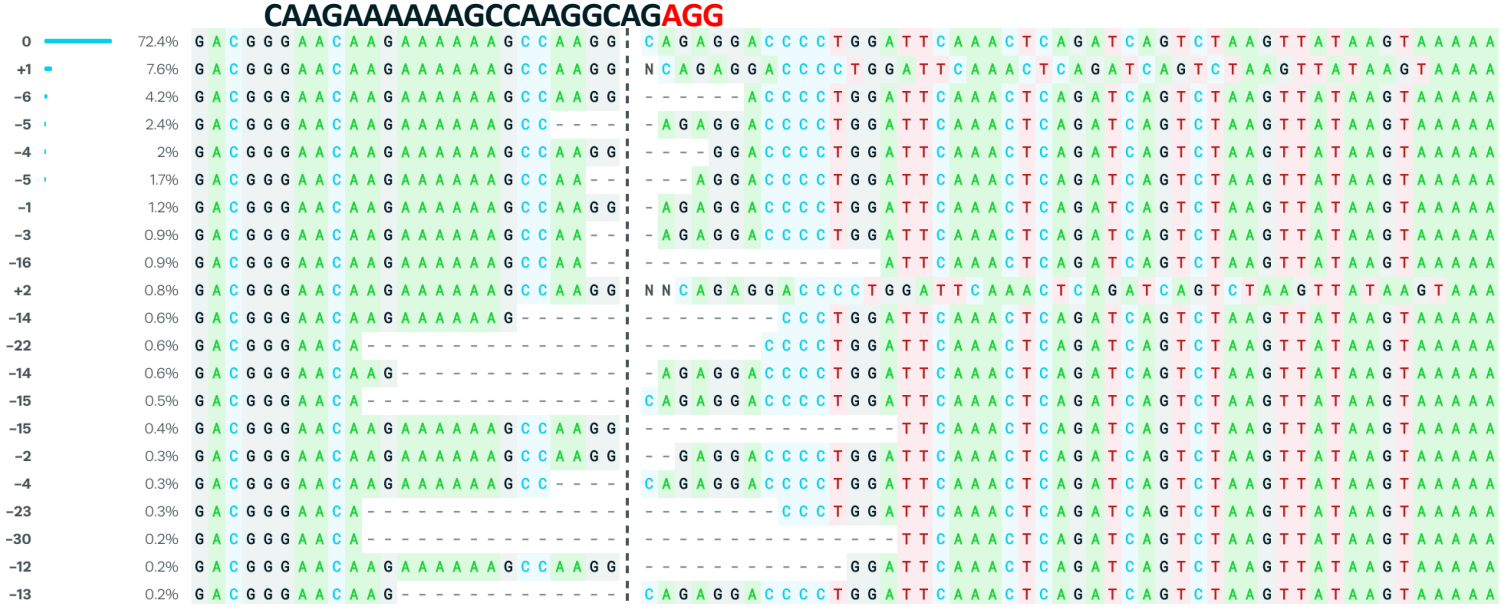
P2 66.7%

CAAGAAAAAAGCCAAGGCACAGG



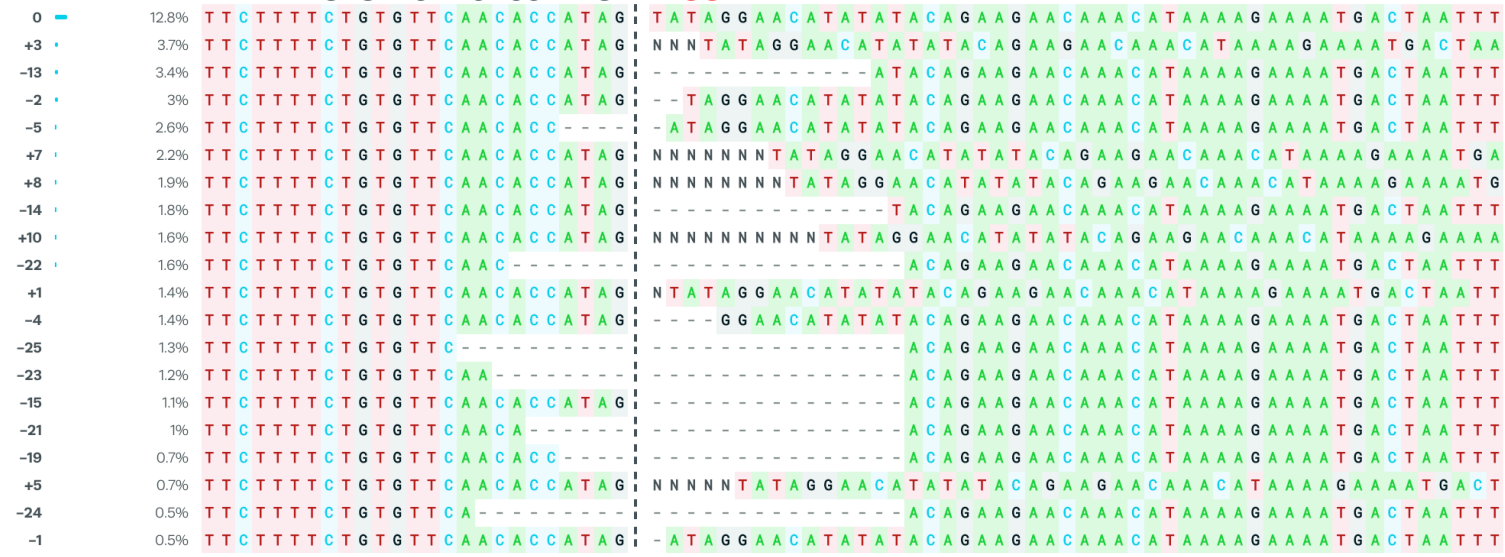
P3 73.7%

CAAGAAAAAAGCCAAGGCAGAGG



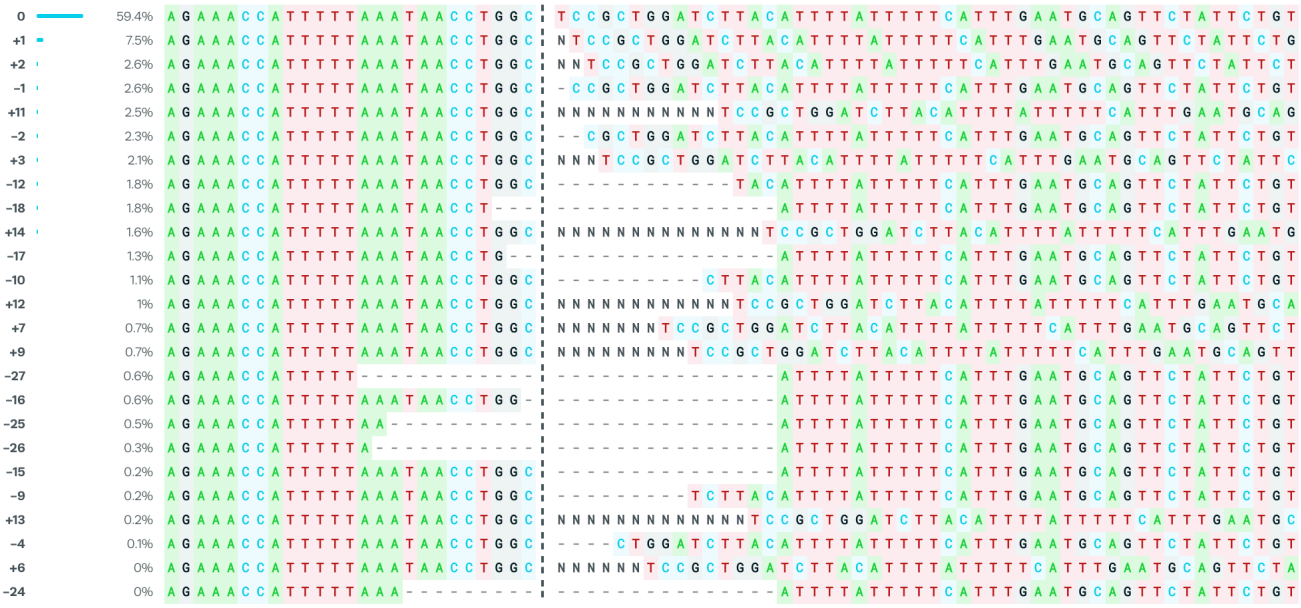
P5 28.8%

TGTGTTCAACACCATAGTATAGG



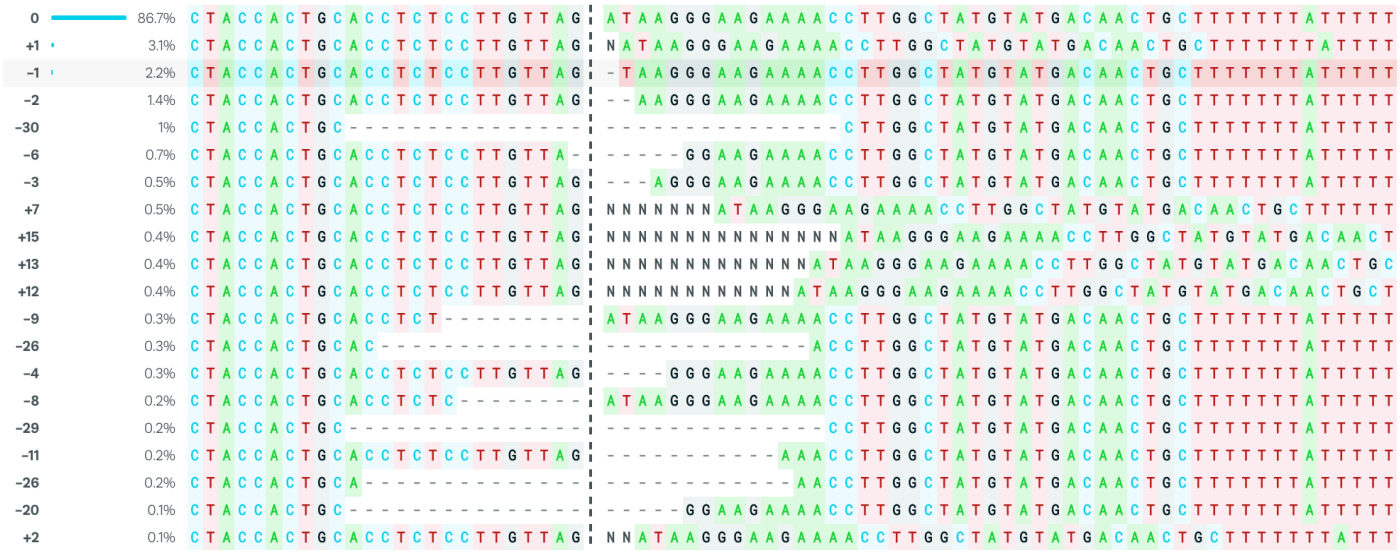
P6 64.8%

ATGTAAGATCCAGCGGAGCCAGG



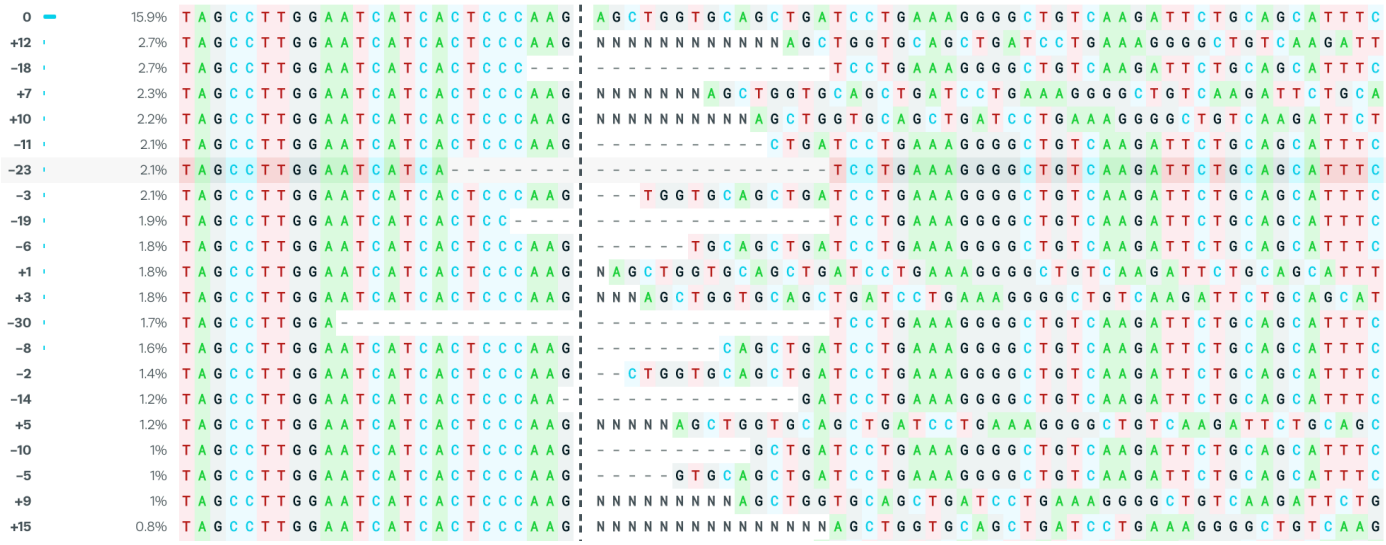
P4 87.4%

GCACCTCTCCTTGTTAGATAAGG



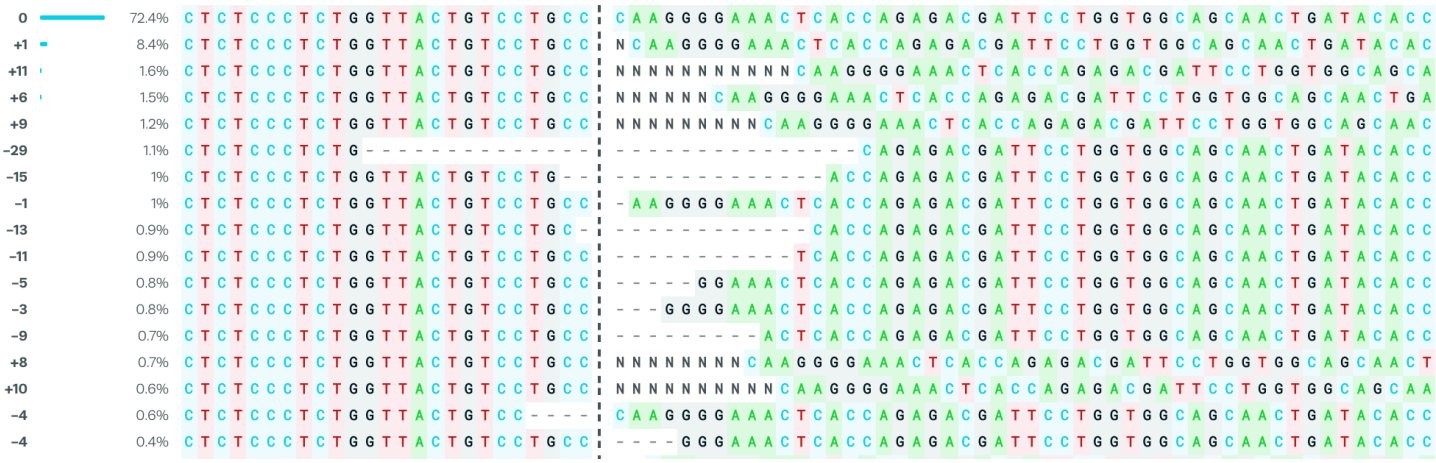
P7 32.1%

GATCAGCTGCACCAGCTCTTGGG



P8 76.5%

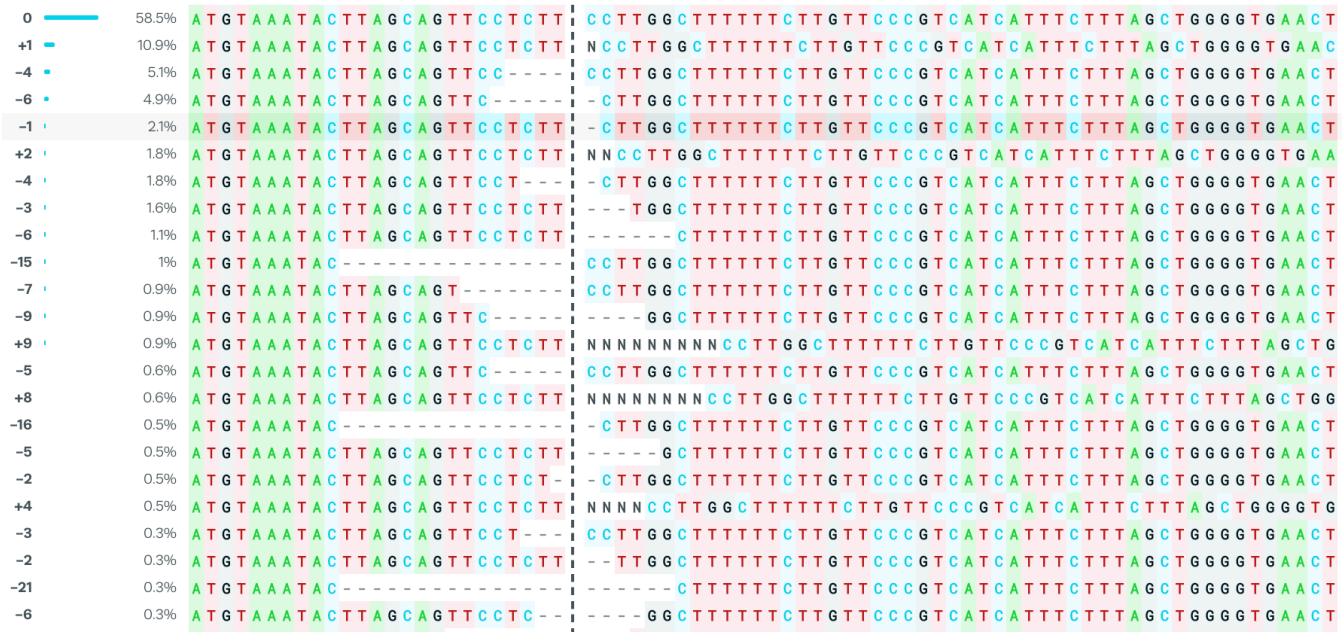
TGGTGAGTTTCCCCTTGGGCAGG



Hela

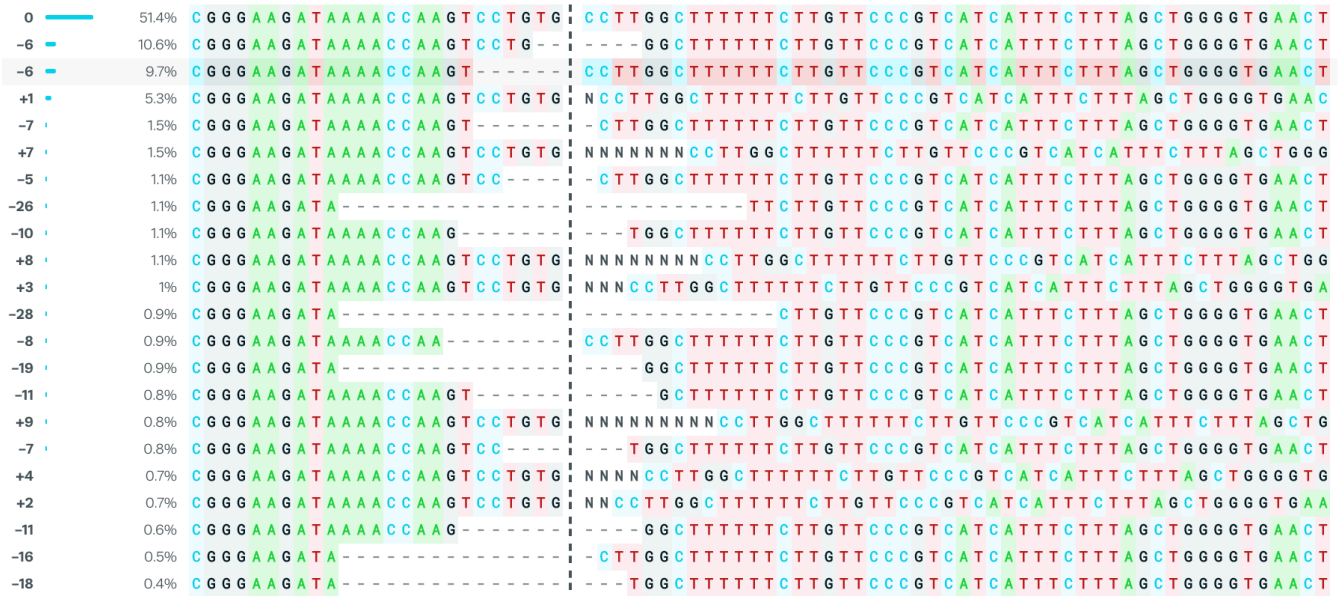
P1 61.1%

CAAGAAAAAAGCCAAGGAAGAGG



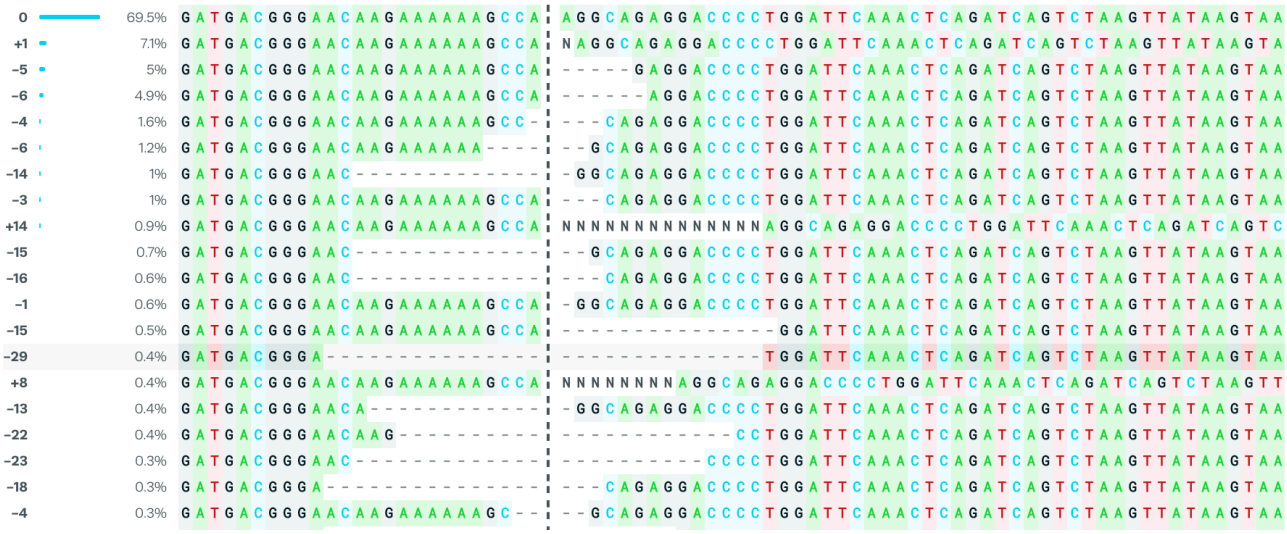
P2 55.0%

CAAGAAAAAAGCCAAGGCACAGG

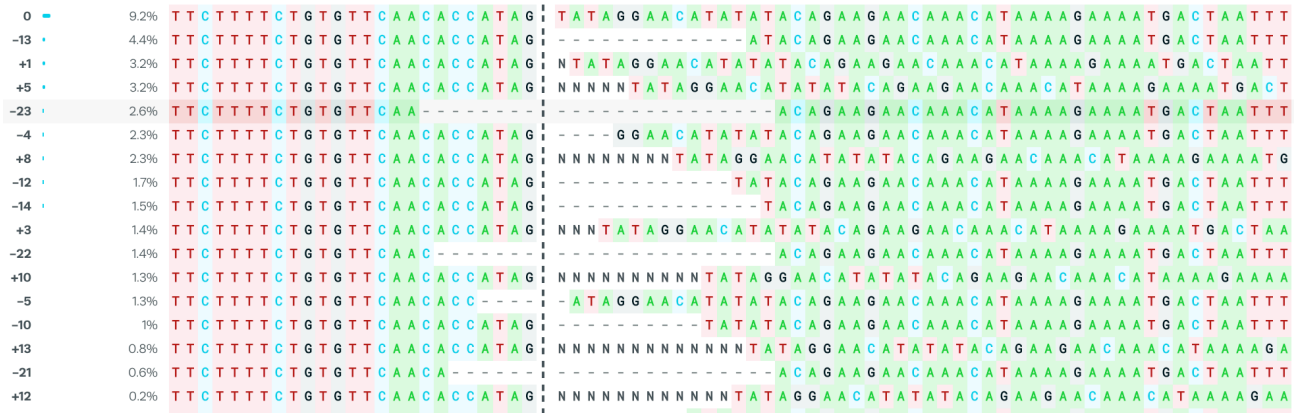


P3 71.6

CAAGAAAAAAGCCAAGGCAGAGG

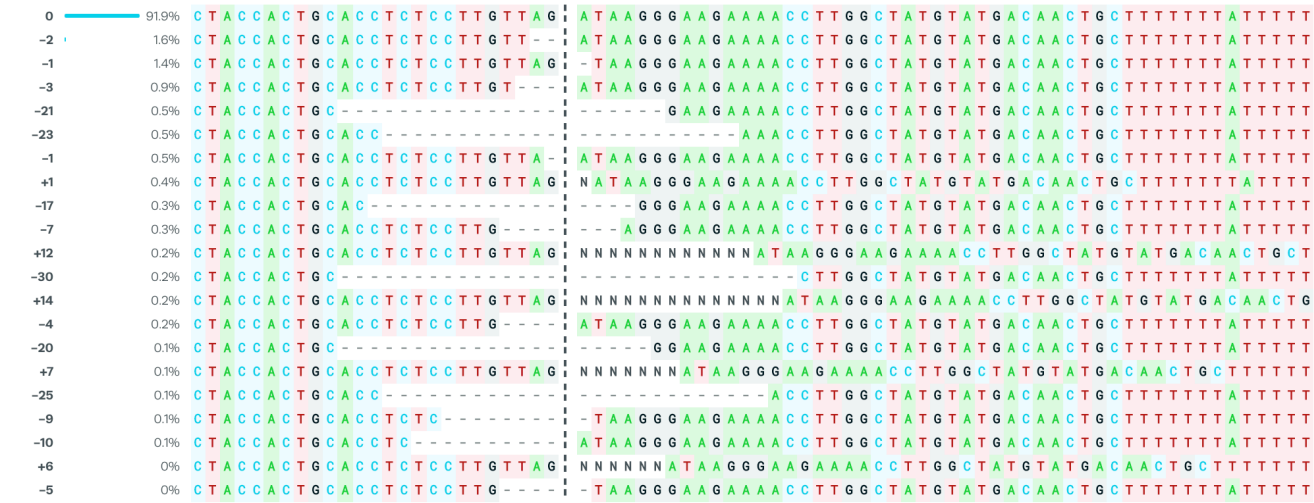


P5 24.0% TGTGTTCAACACCATAGTATAGG

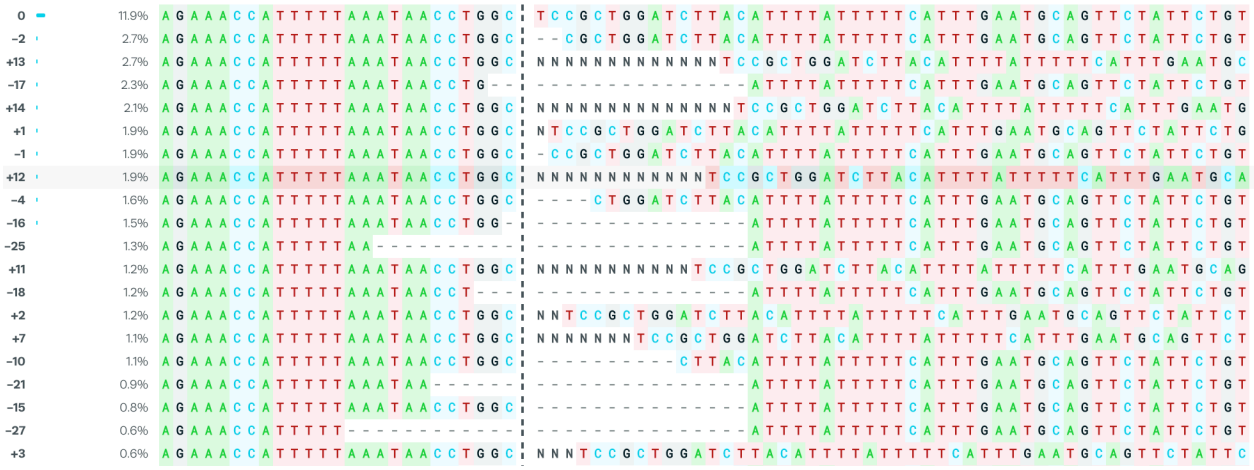


P4 92.3%

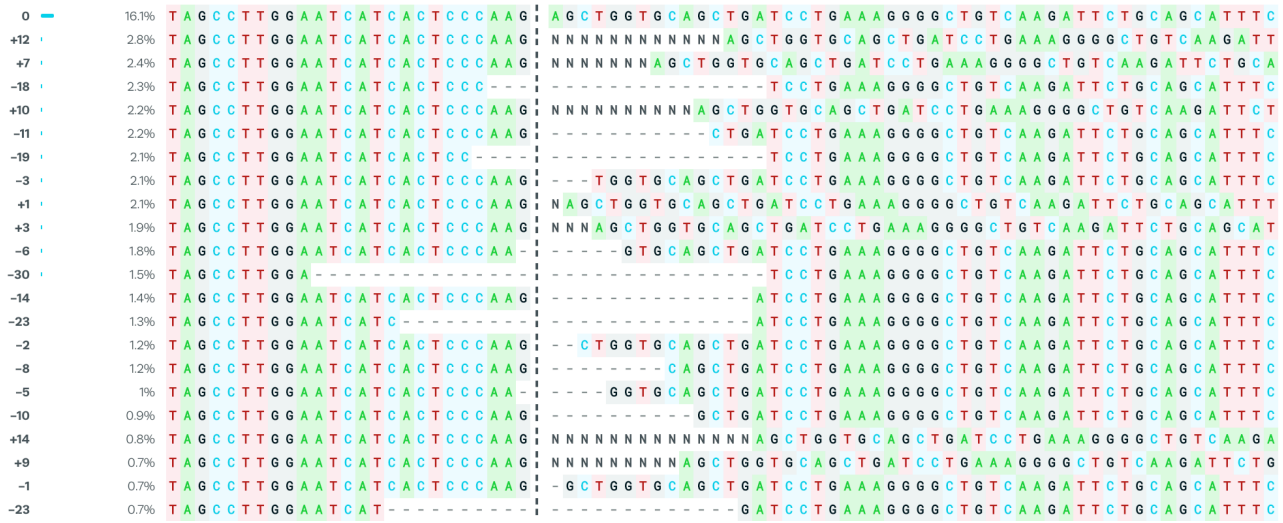
GCACCTCTCCTTGTTAGATAAGG



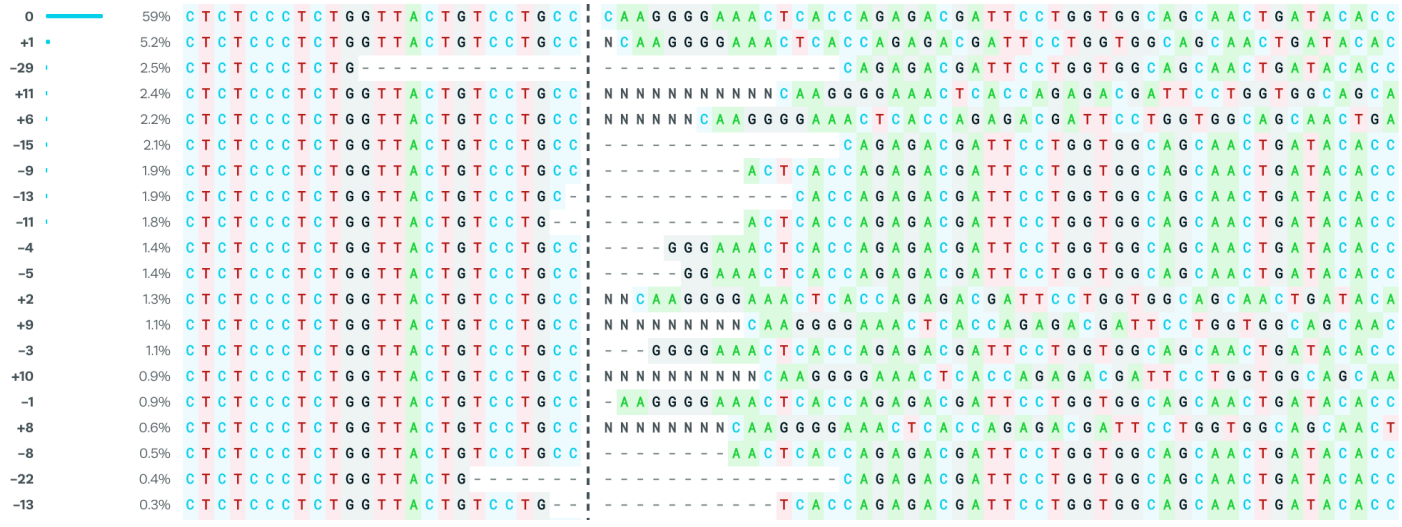
P6
29.4% ATGTAAGATCCAGCGGAGCCAGG



P7 32.6% GATCAGCTGCACCAGCTCTTGGG



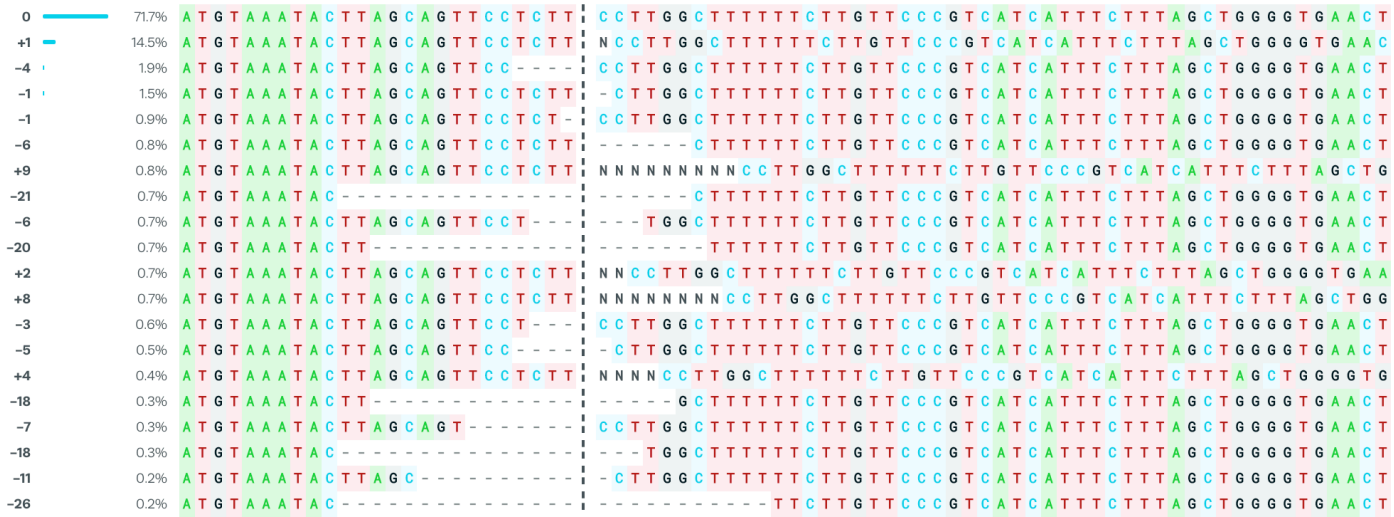
P8
66.4% TGGTGAGTTTCCCCTTGGGCAGG



HepG2

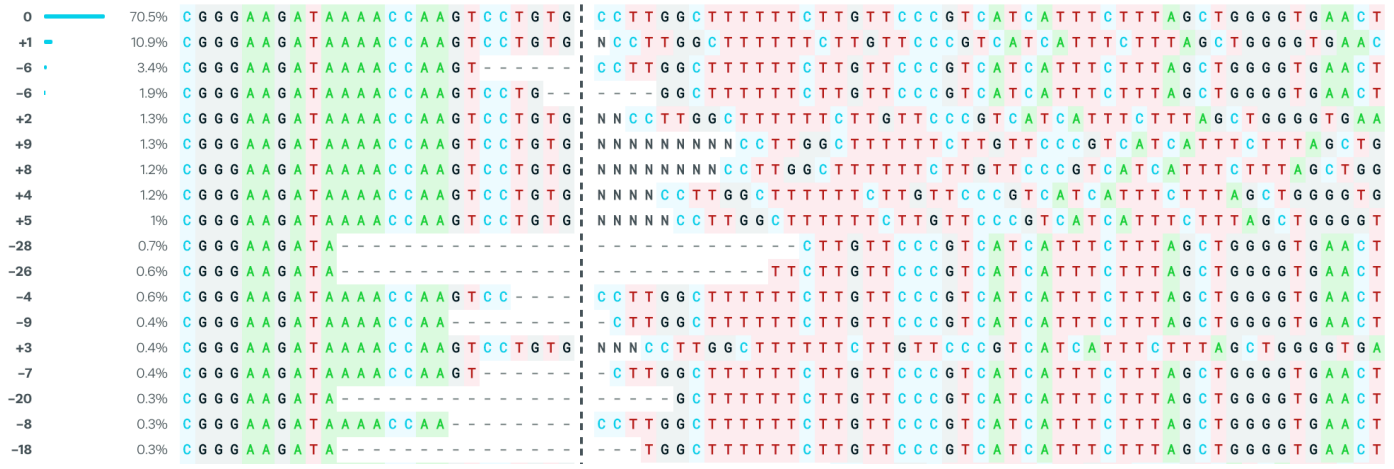
P1 72.9%

CAAGAAAAAAGCCAAGGAAGAGG



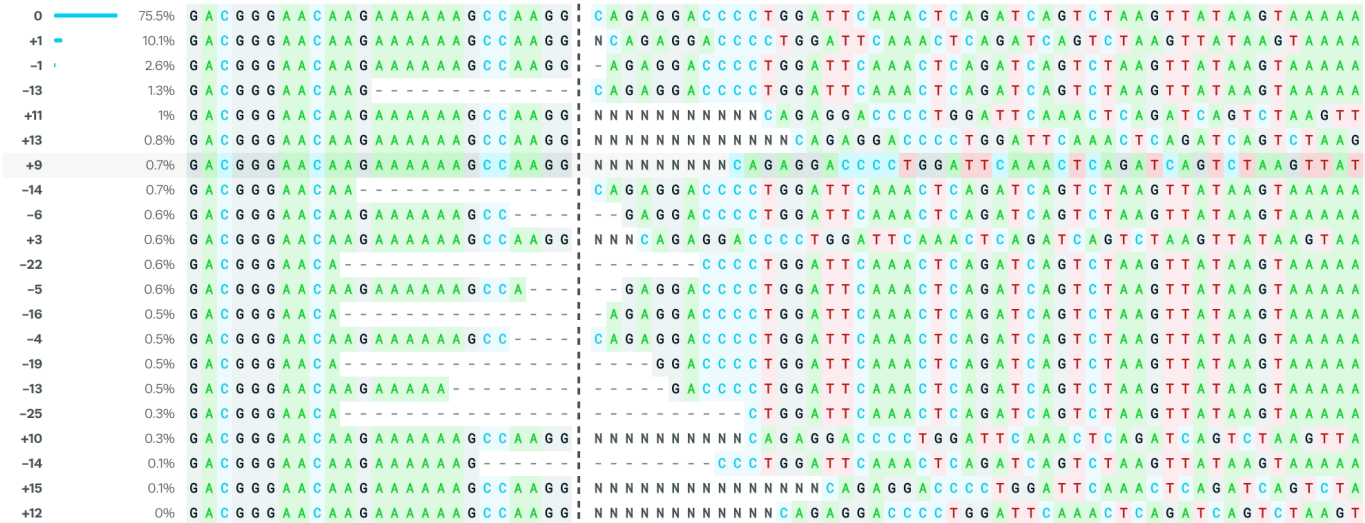
P2 72.9%

CAAGAAAAAAGCCAAGGCACAGG

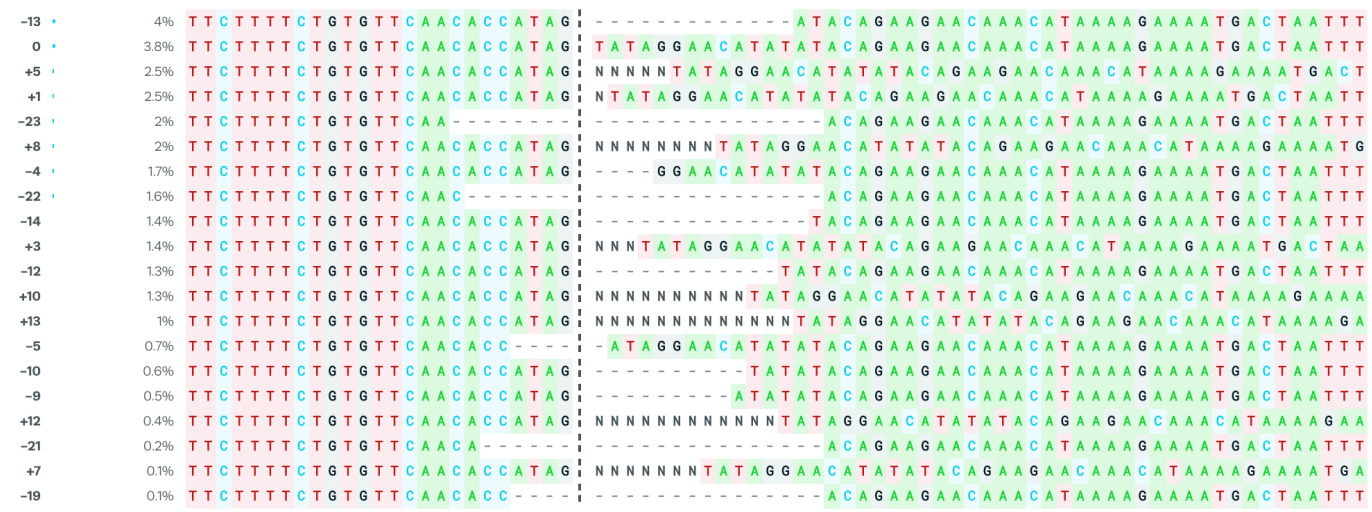


P3 77.1%

CAAGAAAAAAGCCAAGGCAGAGG



P5 13.7%



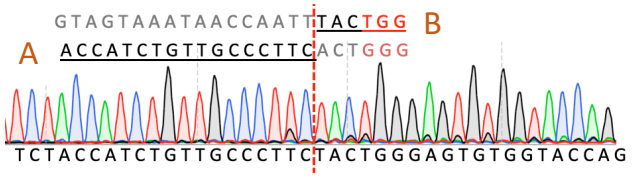
P4 94.3%

P6 21.4%

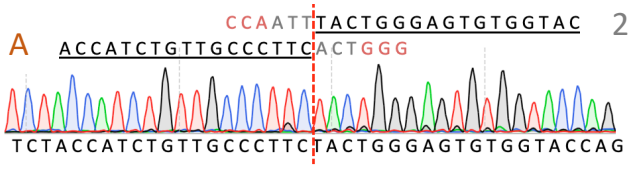
Figure S2

ICE analysis results of repair outcomes of 8 loci after SpCas9 and dual gRNAs cleavage in three human cell lines (HEK293T, Hela and HepG2). The estimated NHBEJ frequency was calculated as the normalized proportion of the blunt end joining indel divided to the sum of significantly deconvoluted indels.

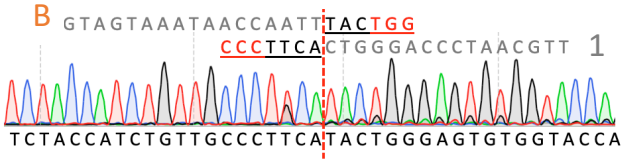
A+B



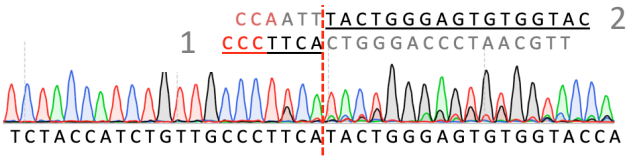
A+2



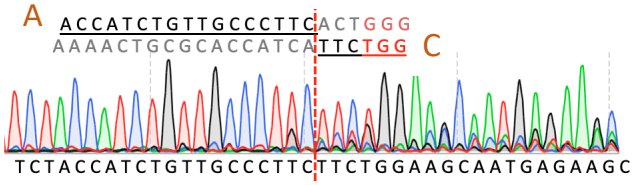
1+B



1+2



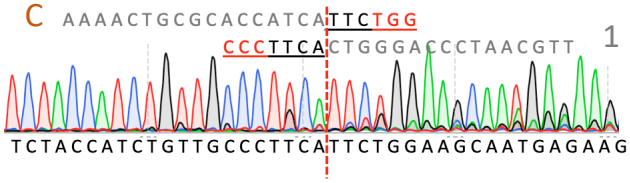
A+C



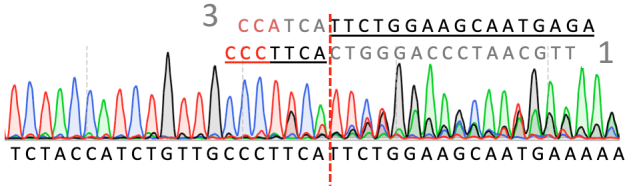
A+3

ND

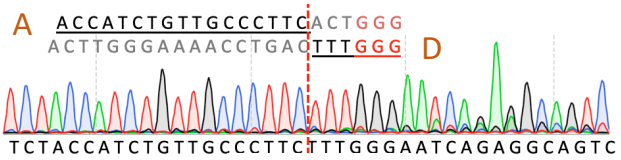
1+C



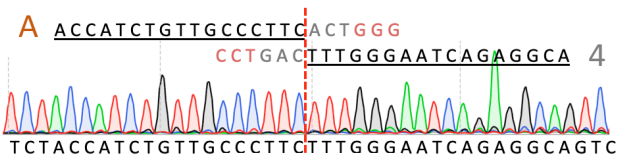
1+3



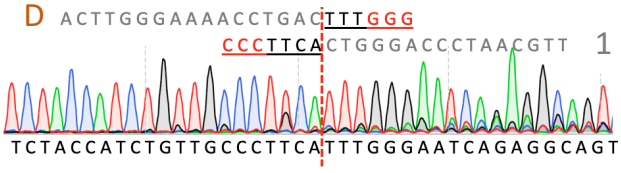
A+D



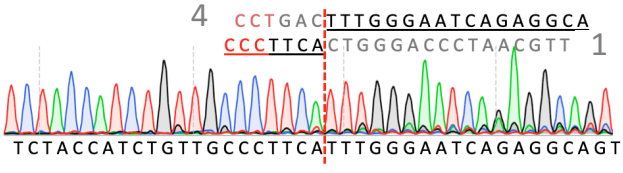
A+4



1+D



1+4



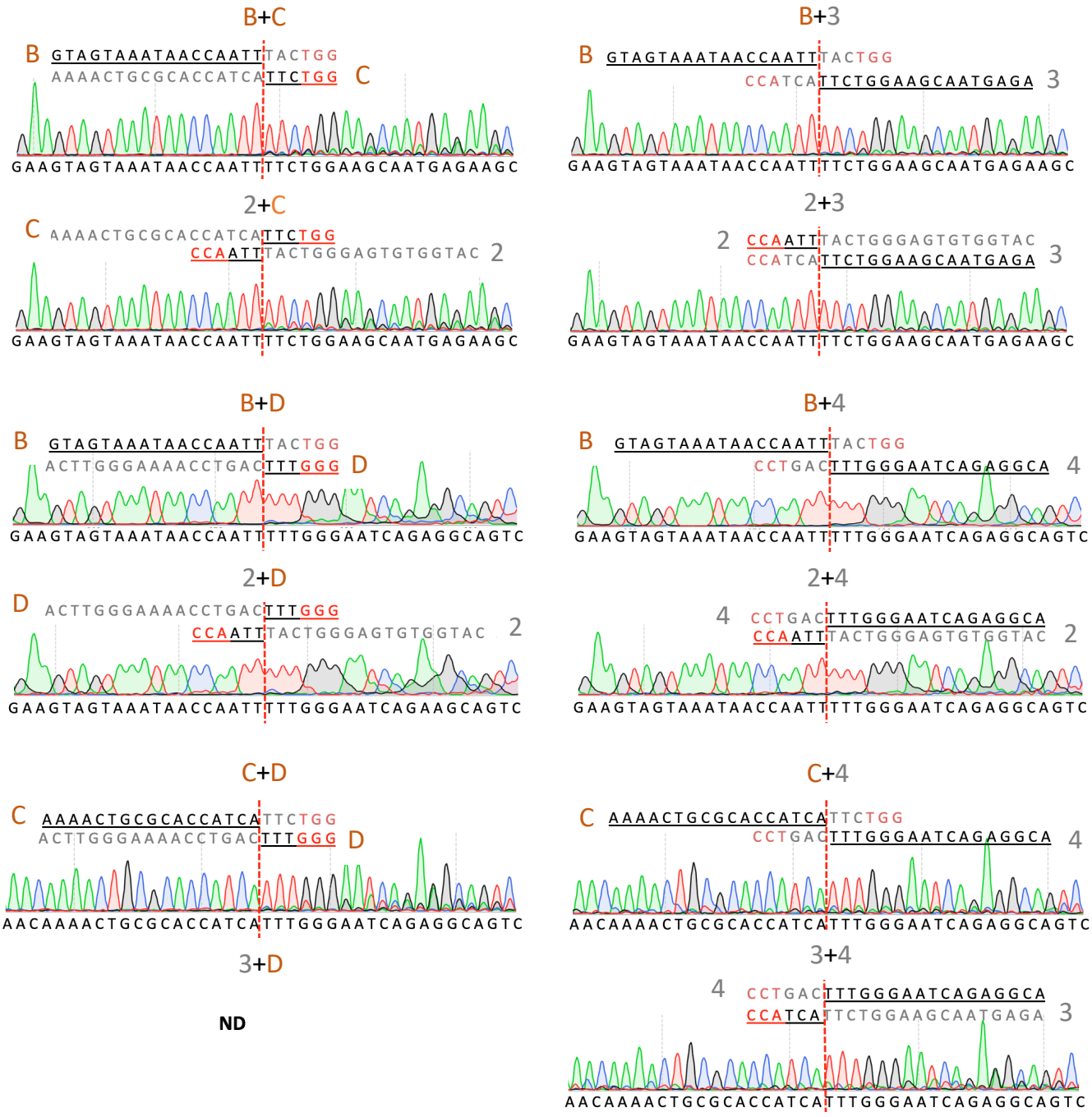


Figure S3

Supporting Sanger sequencing chromatograms of the deletion PCR productions shown in Figure 4B (highlighted with asterisks). ND, Sanger sequencing results not available due to poor signals.

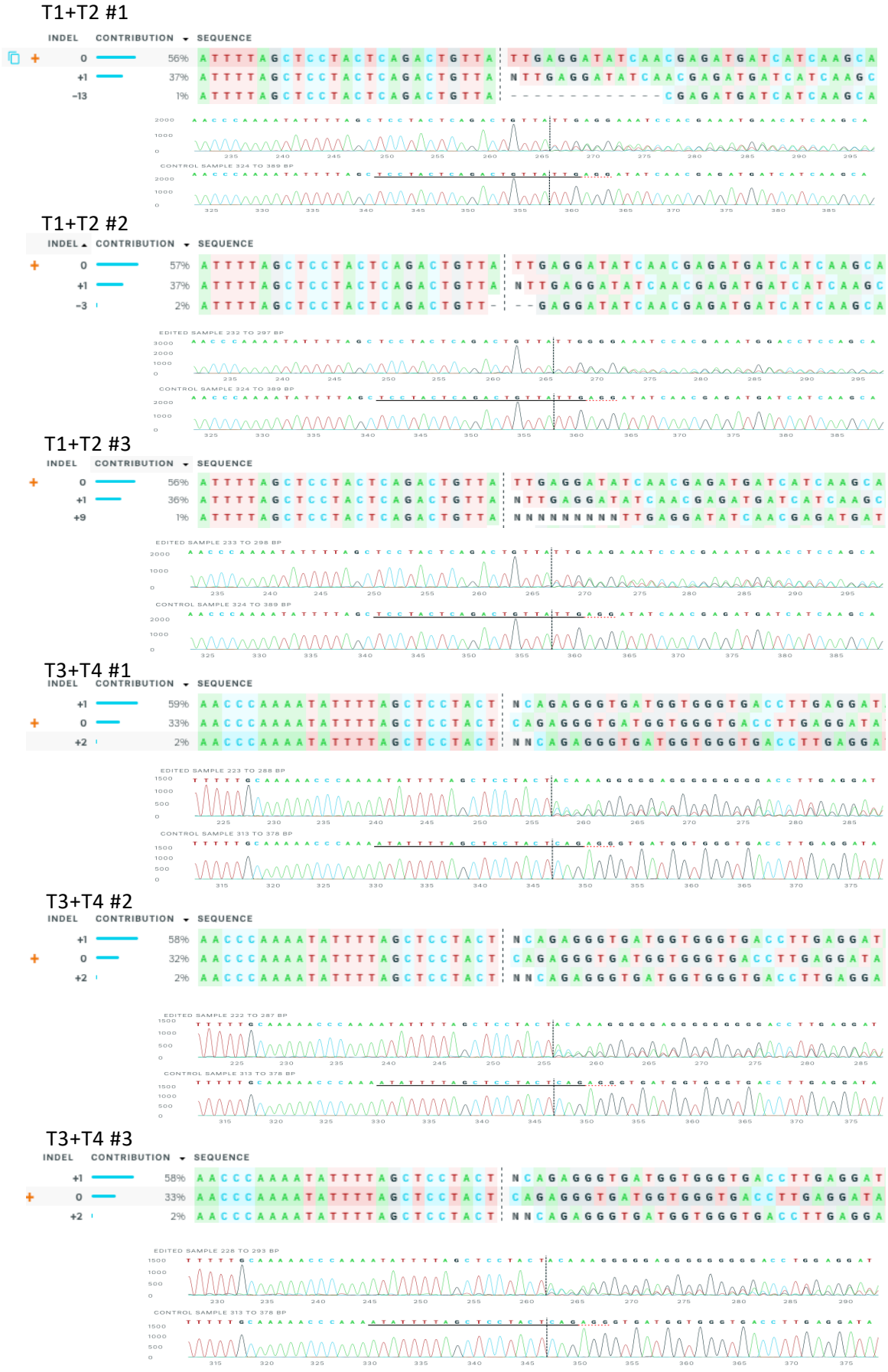
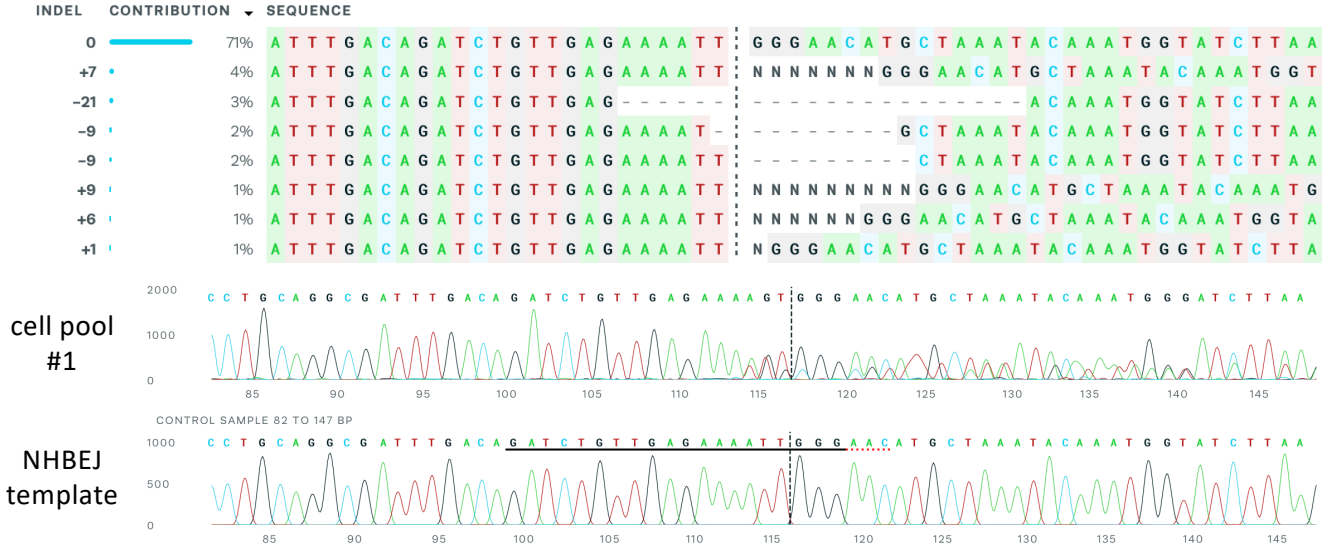
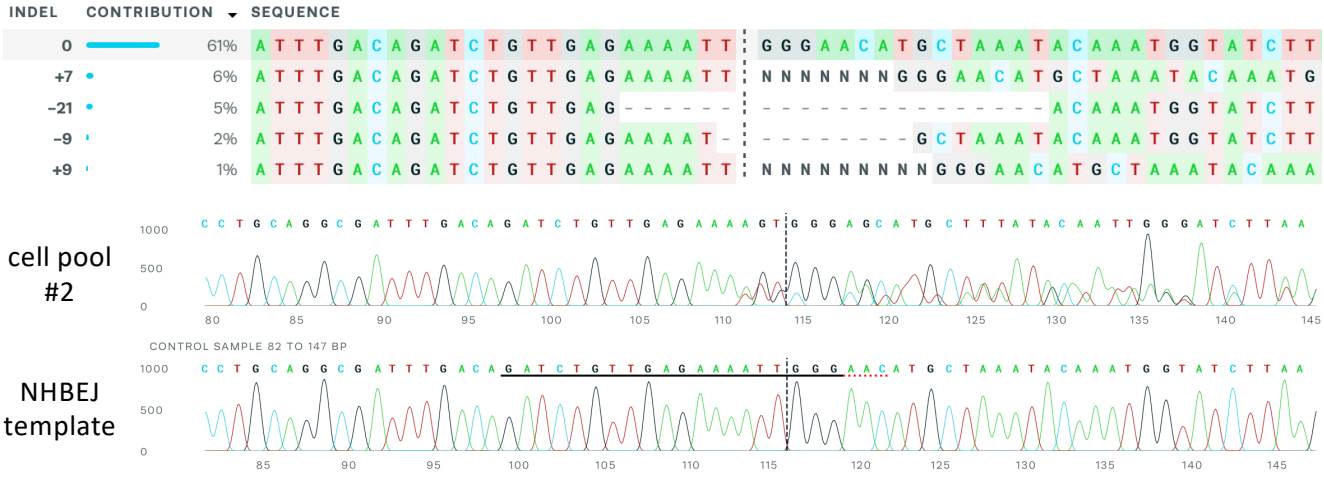
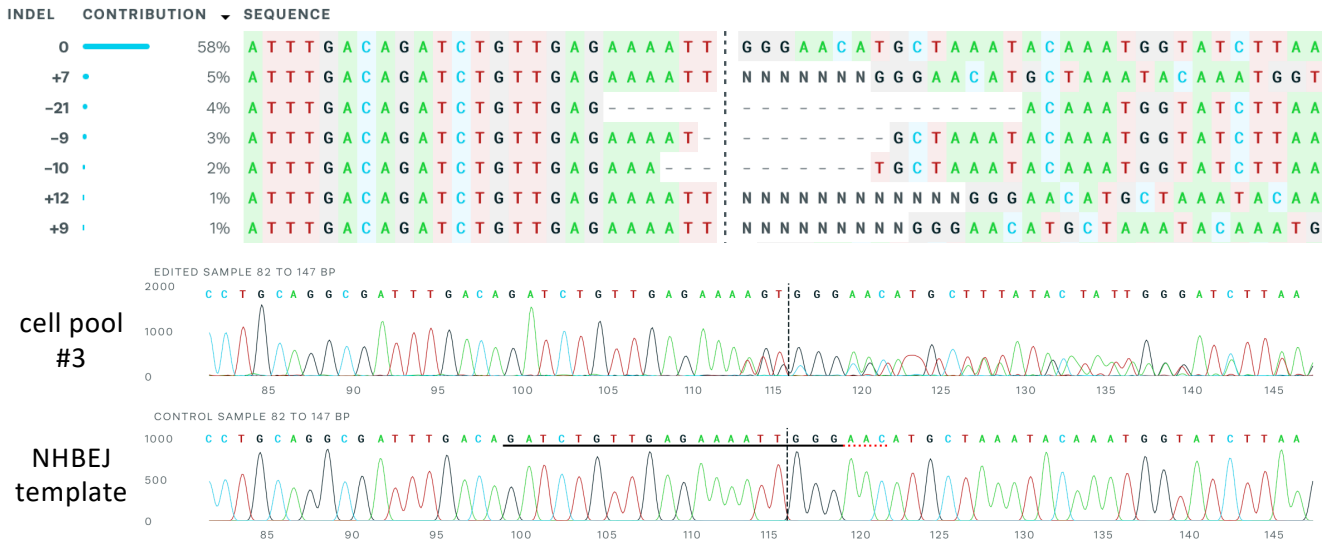


Figure S4
Supporting Sanger sequencing and ICE analysis results of the deletion PCR products of DMD exon 51 from the pool of CRISPR edited HEK293T cells in Figure 5c.

NHBEJ efficiency of gR1+3 nucleofected cell pools







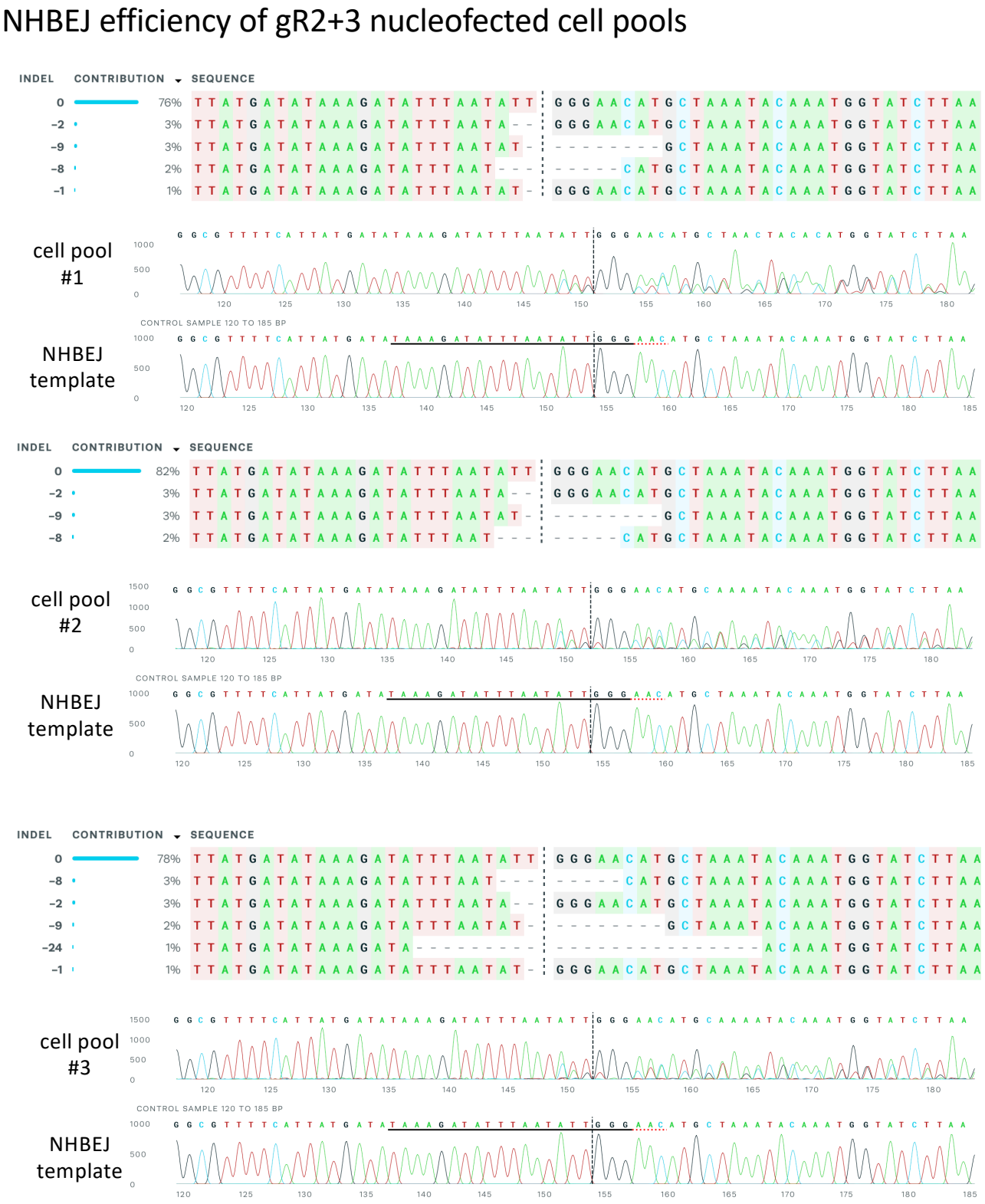
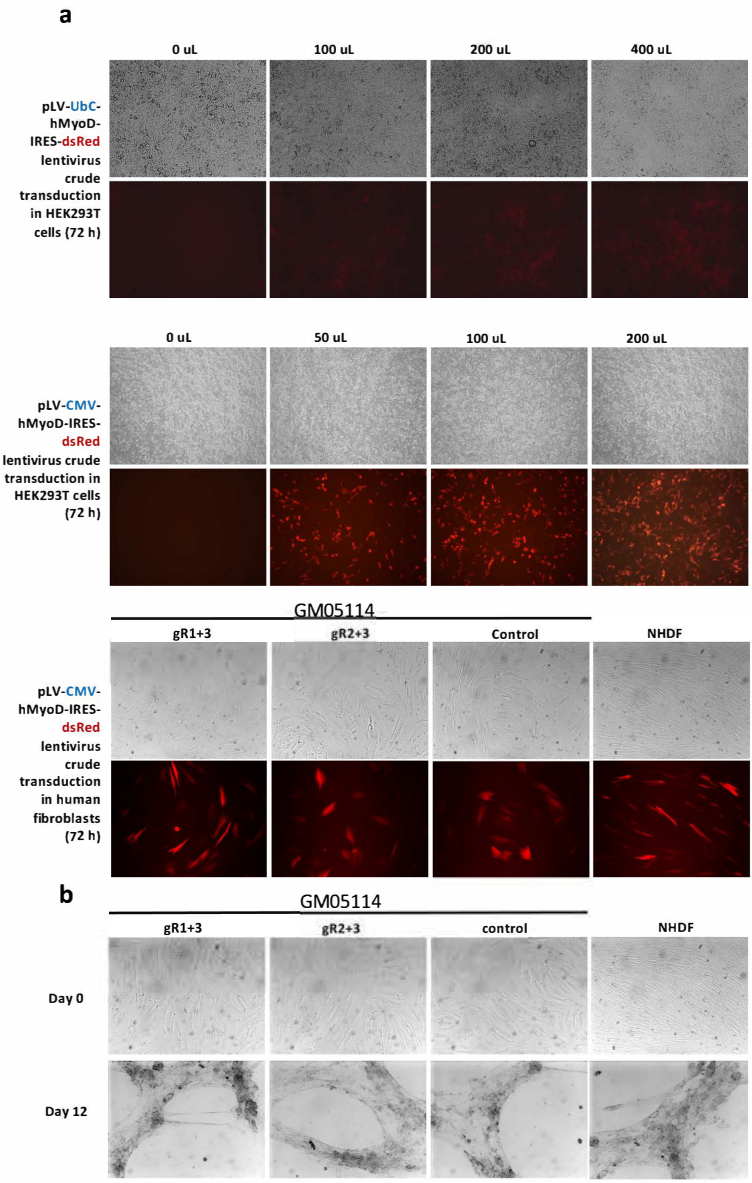
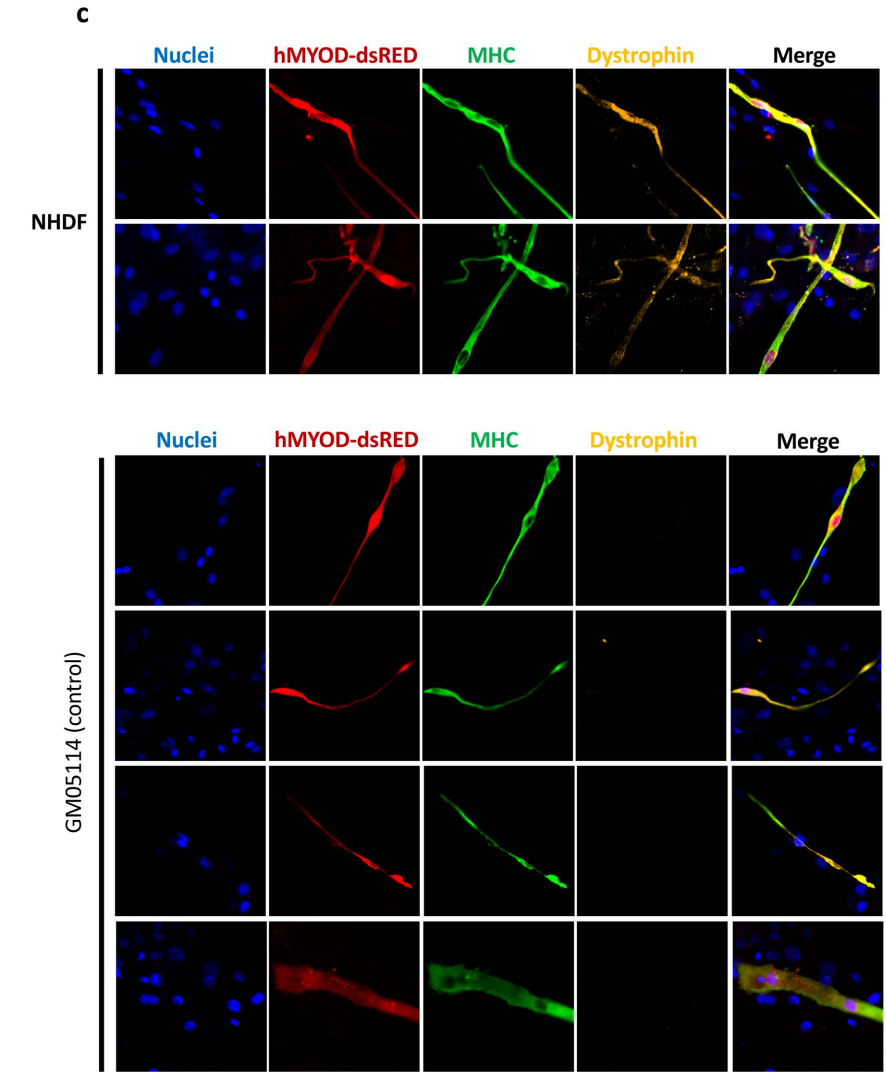


Figure S5
Supporting Sanger sequencing and ICE analysis results of the deletion PCR products of DMD exon 44 from the pool of CRISPR edited DMD ex45del fibroblast cells in Figure 6b.


a
0 uL
100 uL
200 uL
400 uL
pLV-UbC-hMyoD-IRES-dsRed lentivirus crude transduction in HEK293T cells (72 h)
0 uL
50 uL
100 uL
200 uL
pLV-CMV-hMyoD-IRES-dsRed lentivirus crude transduction in HEK293T cells (72 h)
GM05114
gR1+3
gR2+3
Control
NHDF
pLV-CMV-hMyoD-IRES-dsRed lentivirus crude transduction in human fibroblasts (72 h)
b
GM05114
gR1+3
gR2+3
control
NHDF
Day 0
Day 12


c
Nuclei
hMYOD-dsRED
MHC
Dystrophin
Merge
NHDF
Nuclei
hMYOD-dsRED
MHC
Dystrophin
Merge
GM05114 (control)

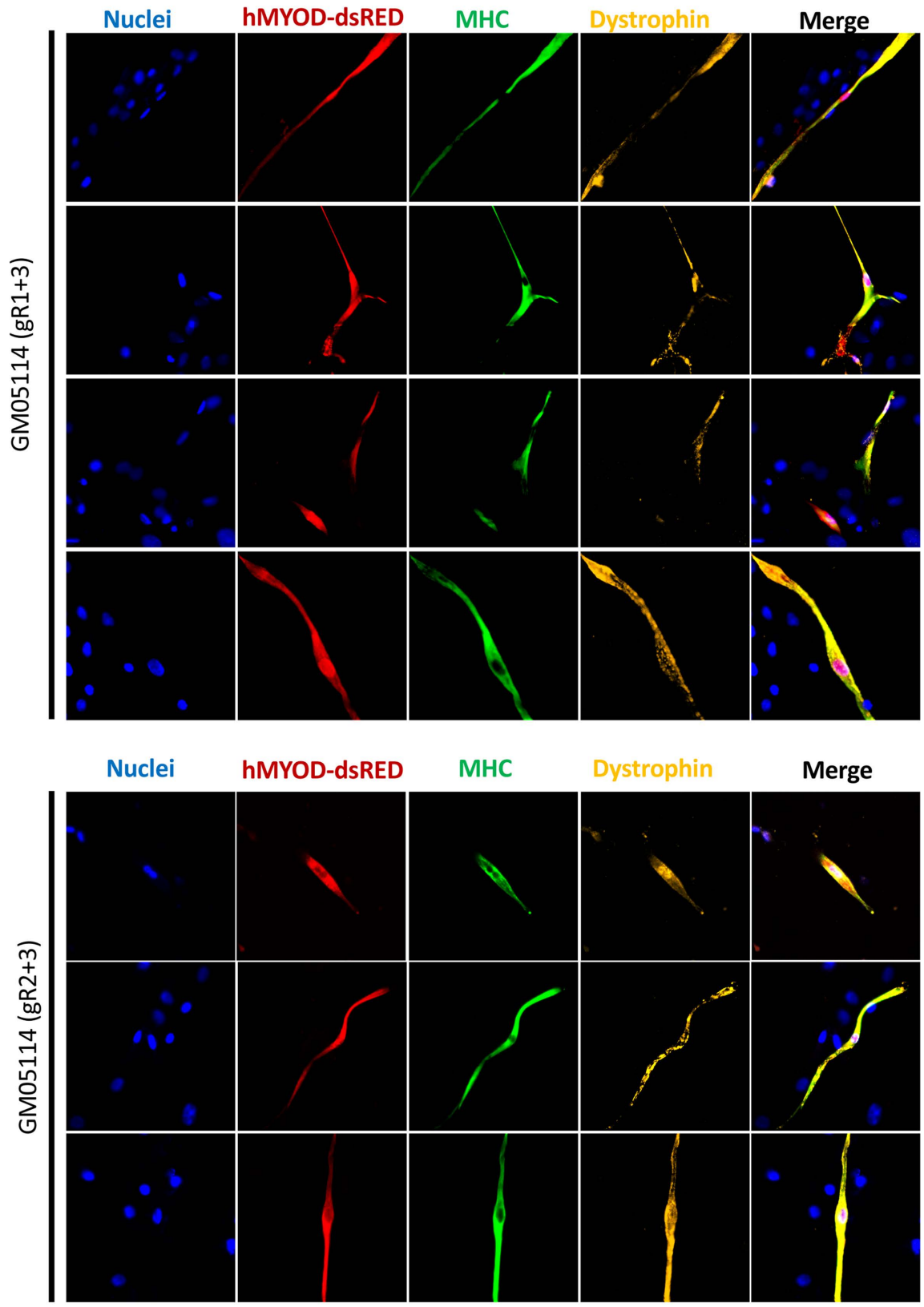
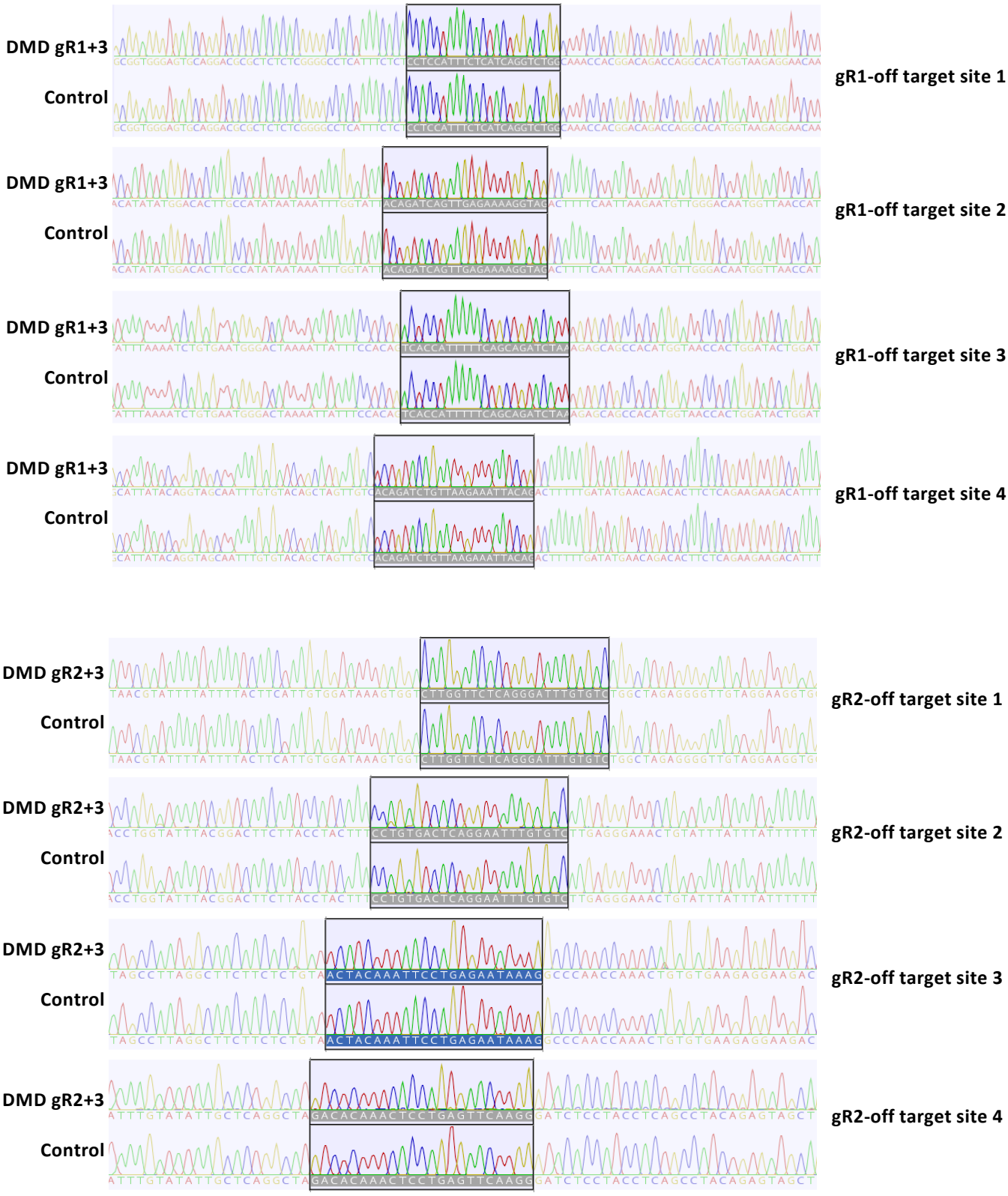


Figure S6
Direct reprogramming and validation of DMD expression in myotubes.
(a) Evaluation of transduction efficiency in HEK293T cells and fibroblasts.
(b) Morphological changes in cell culture before and after direct reprogramming.
(c) Extended representative fluorescence immunostaining images stained with antibodies again MHC and dystrophin. MYOD expression is detected with dsRED. Nuclear, DAPI. Magnification, 40X.


DMD gR1+3
Control
gR1-off target site 1
DMD gR1+3
Control
gR1-off target site 2
DMD gR1+3
Control
gR1-off target site 3
DMD gR1+3
Control
gR1-off target site 4
DMD gR2+3
Control
gR2-off target site 1
DMD gR2+3
Control
gR2-off target site 2
DMD gR2+3
Control
gR2-off target site 3
DMD gR2+3
Control
gR2-off target site 4

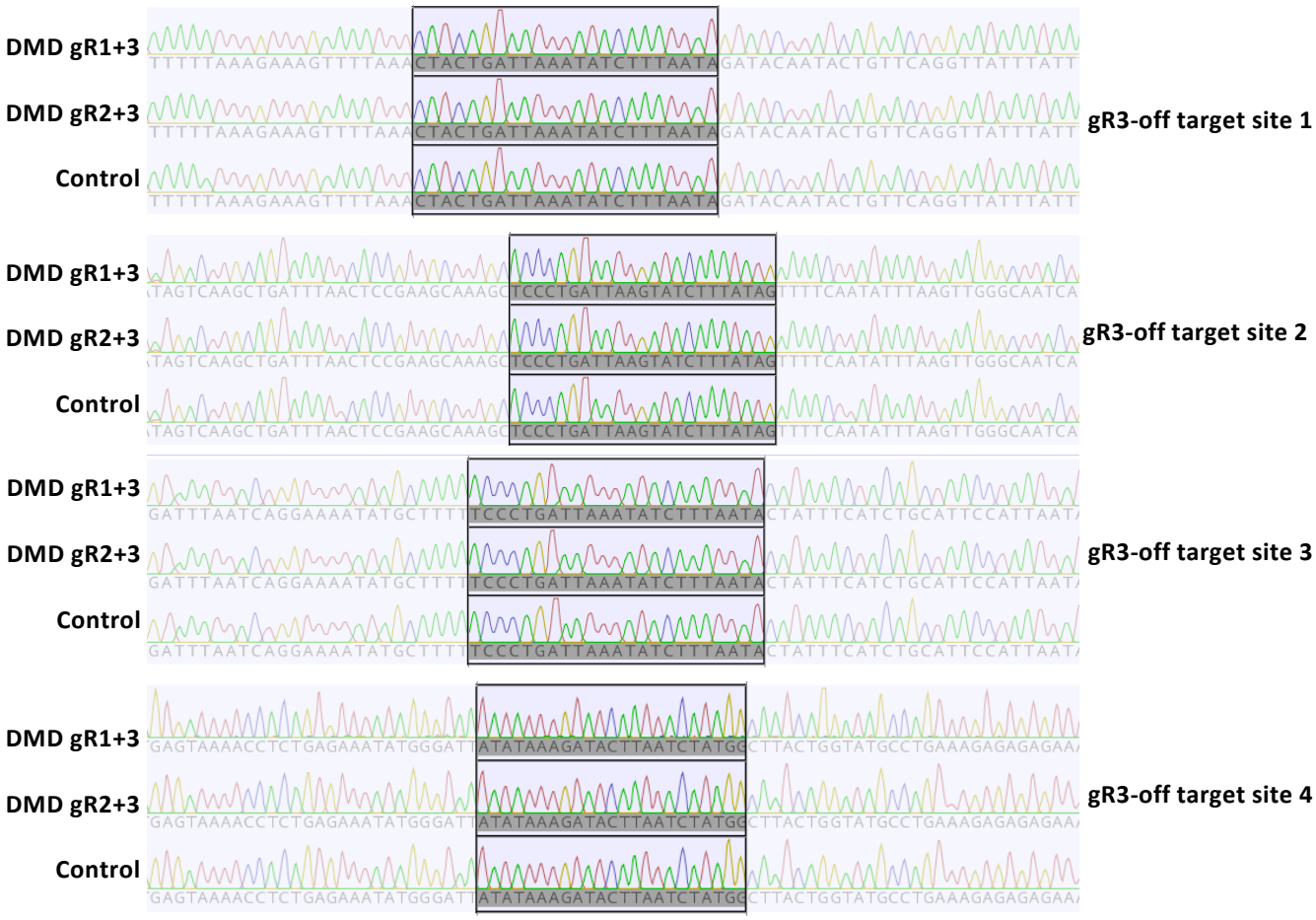


Figure S7
Sanger sequencing chromatograms results for validation of the top 4 predicted off-target sites for the three DMD exon 44 targeting gRNAs. Genome DNA used for Sanger sequencing are from the CRISPR-edited DMD ex45del fibroblasts. Off-target sites are predicted with CRISPRspec.

CRISPR-B	gRNA name	gRNA sequence	Target locus
P1	P1-gRNA1	CCAAGGTTAAGGGCACTTCAGAA	Upstream of *TTR*
	P1-gRNA2	CCCTTTGCATCCAGCAGAAGAGG	*TTR* intron
P2	P2-gRNA1	CCAAGGTTAAGGGCACTTCAGAA	Upstream of *TTR*
	P2-gRNA2	TTGCCAAAGAACCCTCCCACAGG	*TTR* intron
P3	P3-gRNA1	CCAAGGTTAAGGGCACTTCAGAA	Upstream of *TTR*
	P3-gRNA2	TGTTTCACAGATAATGGCAGAGG	Downstream of *TTR*
P4	P4-gRNA1	GCACCTCTCCTTGTTAGTAGGG	*CREB* intron
	P4-gRNA2	GTAAATGGTGCTCTCAGATAAGG	*CREB* intron
P5	P5-gRNA1	CCATAGGTATCTATGCCAGCAGC	*CREB* intron
	P5-gRNA2	TGTATAGTCACCTTATGTATAGG	*CREB* intron
P6	P6-gRNA1	ATGTAAGATCCAGCGGACATAGG	*CREB* intron
	P6-gRNA2	TGCACTCCAACCAGTTAGCCAGG	*CREB* intron
P7	P7-gRNA1	CCAGCTTTACTCGCACAGCCTCC	*STAT2* intron
	P7-gRNA2	CCAGGCAGGAAGCTGCACTTGGG	*STAT2* intron
P8	P8-gRNA1	CCCTTGTCCAACCACTGCTAGAC	*IRF9* intron
	P8-gRNA2	AACTGGGTGGGCCTAAGGGCAGG	*IRF9* intron
***DMD*-int44-R1**	A	ACCATCTGTTGCCCTTCACTGGG	*DMD* intron 44
	1	CCCTTCACTGGGACCCTAACGTT	*DMD* intron 44
***DMD*-int44-R2**	B	GTAGTAAATAACCAATTTACTGG	*DMD* intron 44
	2	CCAATTTACTGGGAGTGTGGTAC	*DMD* intron 44
***DMD*-int44-R3**	C	AAAACTGCGCACCATCATTCTGG	*DMD* intron 44
	3	CCATCATTCTGGAAGCAATGAGA	*DMD* intron 44
***DMD*-int44-R4**	D	ACTTGGGAAAACCTGACTTTGGG	*DMD* intron 44
	4	CCTGACTTTGGGAATCAGAGGCA	*DMD* intron 44
***DMD*-EX51 NHBEJ-gRNAs**	*DMD*exon51-T1	TCCTACTCAGACTGTTACTCTGG	*DMD* exon 51
	*DMD*exon51-T2	GGTGATGGTGGGTGACCTTGAGG	*DMD* exon 51
	*DMD*exon51-T3	ACCAGAGTAACAGTCTGAGTAGG	*DMD* exon 51
	*DMD*exon51-T4	ATCAAGTTATAAAATCACAGAGG	*DMD* exon 51
DMD EX44 NHBEJ gRNAs (Synthesized)	*DMD*exon44-gR1	acagatctgttgagaaatggCGG	*DMD* exon 44
	*DMD*exon44-gR2	atataaagatatttaatcagTGG	*DMD* exon 44
	*DMD*exon44-gR3	gacacaaattcctgagaattGGG	*DMD* exon 44

Table S1. gRNAs used in this study

Primer name	5’-3’ sequence	Wildtype length (bp)	CRISPR-B length (bp)
P1-F	GGGTGATGGTGATCACACCACT	31690	486
P1-R	GGTTACAGGACTATTCTAAGGG		
P2-F	GGGTGATGGTGATCACACCACT	30855	473
P2-R	CATTTAGGGGCAGACAGTAGAG		
P3-F	GGGTGATGGTGATCACACCACT	58658	479
P3-R	CTGAGAAATACGTGCTGGAGAA		
P4-F	AACGGGCTGATTTTGTCCTAC	1281	581
P4-R	CCACCTTTCTCATTCCTATC		
P5-F	CCACCTTTCTCATTCCTATC	812	664
P5-R	CTCCCAAACACTTCCACT		
P6-F	TGTCGTGGCAAGAGTCTACT	1084	439
P6-R	TGTCCGTAACATGGTATTCTTAGA		
P7-F	ATTTGTTCCCGTCTCCCT	773	547
P7-R	AGAATATGCACCAAACTGA		
P8-F	CAGCTAAGACCATGTTCCGG	707	329
P8-R	GGTCCAGCTGTCTGGAAGAC		
DMDintron44For	TAGGATACACCTAACATGGCAATC	See table S3	See table S3
DMDintron44Rev	TGGTATTCTGGGATATACGACCAC		
DMD-R1-1	ATGCCATGCTGGACAACGGAAG		
gR1-F	ACCATCTGTTGCCCTTCACT		
DMD-R2-1	ACACGAAGATCAATATGGCTGG		
DMD-exon51-F	ACTTGTCCAGGCATGAGAATGAG	667	See figure 5
DMD-exon51-R	TATACTTAGGCTGAATAGTGAGAG		
DMD-exon44-F	TGCAGGAAACTATCAGAGTGAT	358	gR1+3: 267 bp
DMD-exon44-R	ATCACCCTTCAGAACCTGATCT		gR2+3: 306 bp
DMD-EX45del-RT-F	GCAAGAAGACAGCAGCATTGCA	552	gR1+3: 376+288 bp
DMD-EX45del-RT-R	CAGGTTCAAGTGGGATACTAGC		gR2+3: 376+324 bp
hGAPDH-RT-F	TGGTATCGTGGAAGGACTCATGAC	189	
hGAPDH-RT-R	ATGCCAGTGAGCTTCCCGTTCAGC		

Table S2. Primers used in this study

DMD-int 44 pair-gRNAs	Primers combination	WT length (bp)	Length after del (bp)
A+B	DMD-For + DMD-R1-1	846	432
A+2	DMD-For + DMD-R1-1	846	432
A+C	DMD-For + DMD-Rev	2431	1634
A+3	DMD-For + DMD-Rev	2431	1634
A+D	DMD-For + DMD-Rev	2431	538
A+4	DMD-For + DMD-Rev	2431	538
1+B	DMD-For + DMD-R1-1	846	432
1+2	DMD-For + DMD-R1-1	846	432
1+C	DMD-For + DMD-Rev	2431	1634
1+3	DMD-For + DMD-Rev	2431	1634
1+D	DMD-For + DMD-Rev	2431	538
1+4	DMD-For + DMD-Rev	2431	538
B+C	gR1-F + DMD-R2-1	937	554
B+3	gR1-F + DMD-R2-1	937	554
B+D	DMD-For + DMD-Rev	2431	952
B+4	DMD-For + DMD-Rev	2431	952
2+C	gR1-F + DMD-R2-1	937	554
2+3	gR1-F + DMD-R2-1	937	554
2+D	DMD-For + DMD-Rev	2431	952
2+4	DMD-For + DMD-Rev	2431	952
C+D	DMD-For + DMD-Rev	2431	1335
C+4	DMD-For + DMD-Rev	2431	1335
3+D	DMD-For + DMD-Rev	2431	1335
3+4	DMD-For + DMD-Rev	2431	1335

Table S3. gRNAs combinations used for PAM direction and cleavage sites influence tests

gRNA	TargetSeq	Mismatches	CRISPRoff	Coordinates	PCR primers-F	PCR primers-R	PCR length
1	ACAGATCTGTTGAGAAATGGCGG	0	0.842361111	chrX:32217032-32217055:-			
	cCAGAcCTGaTGAGAAATGGAGG	3	0.436805556	chr14:105469449-105469472:-	TCTCTTGCTGGCCAGAGAGCTG	TGGCCAGGCCTCTTCCCAACAC	324 bp
	ACAGATCaGTTGAGAAAaGGTAG	2	0.370833333	chr1:110926988-110927011:+	GCCAACAGATGGCATTATGG	GAAGTTCAGATCCCTGGAGC	566 bp
	ttAGATCTGcTGAaAAATGGTGA	4	0.360416667	chr1:213519718-213519741:-	GCAGCTGCGTGGTACTAGAGAC	GGCAGAAGCTGTGTGATCTCTC	382 bp
	ACAGATCTGTTaAGAAATtaCAG	3	0.354861111	chr15:49317220-49317243:+	GCTGGGAGTATCCTGAGAAGTG	AGGAATCGCCACACTGTCTTCC	386 bp
2	ATATAAAGATATTTAATCAGTGG	0	0.5375	chrX:32216996-32217019:-			
	tatTAAAGATATTTAATCAGTAG	3	0.294444444	chrX:27845602-27845625:-	GGTATTAGAGTGATGATAGCC	ATTGGCCTCTAGCCAGGATGAT	432 bp
	cTATAAAGATAcTTAATCAGGGA	2	0.25625	chr11:34402257-34402280:-	CCTGTGTATGGATAGTTTGGAG	AGTCCTGTACAGAGCATGGCTA	397 bp
	tatTAAAGATATTTAATCAGGGA	3	0.268055556	chr12:69493880-69493903:-	TGGCAAATGATAGCACTTGTC	TGTAGCCTCAGCGTCTAGCAC	407 bp
	tatTAAAGATAcTTAATCAGAAG	4	0.234027778	chr6:87404961-87404984:+	GGTTGGTCTCAAACTCCTGACC	GAAGGCATGAGAACTCTTCTCAG	437 bp
3	GACACAAATTCCTGAGAATTGGG	0	0.761111111	chrX:32216944-32216967:-			
	GACACAAATcCCTGAGAAccAAG	3	0.472916667	chr6:483275-483298:-	CCTCACTGCTATTTAAGCCCAG	CTAGACAAAGTGGGCAGTGAAG	362 bp
	GACACAAATTCCTGAGtcacAGG	4	0.438194444	chr21:41452561-41452584:-	CATCTGTGGCTCAAGCCTCCTG	CTCCATGGAAGAGTCTGTTGCC	432 bp
	actACAAATTCCTGAGAATaAAG	4	0.398611111	chr7:43654891-43654914:+	TGTAGAACATGTTCCAGTTCCG	AATTGGTCTCAGTGCATGCTGC	326 bp
	ccCACAAAcTCCTGAGAATaCGG	4	0.390277778	chr2:52810266-52810289:+	GGAGATAACCACAATGAGACTG	CAGAACAGCATACAGGTTGAC	435 bp

Table S4, top 4 predicted potential off-targets by CRISPRspec