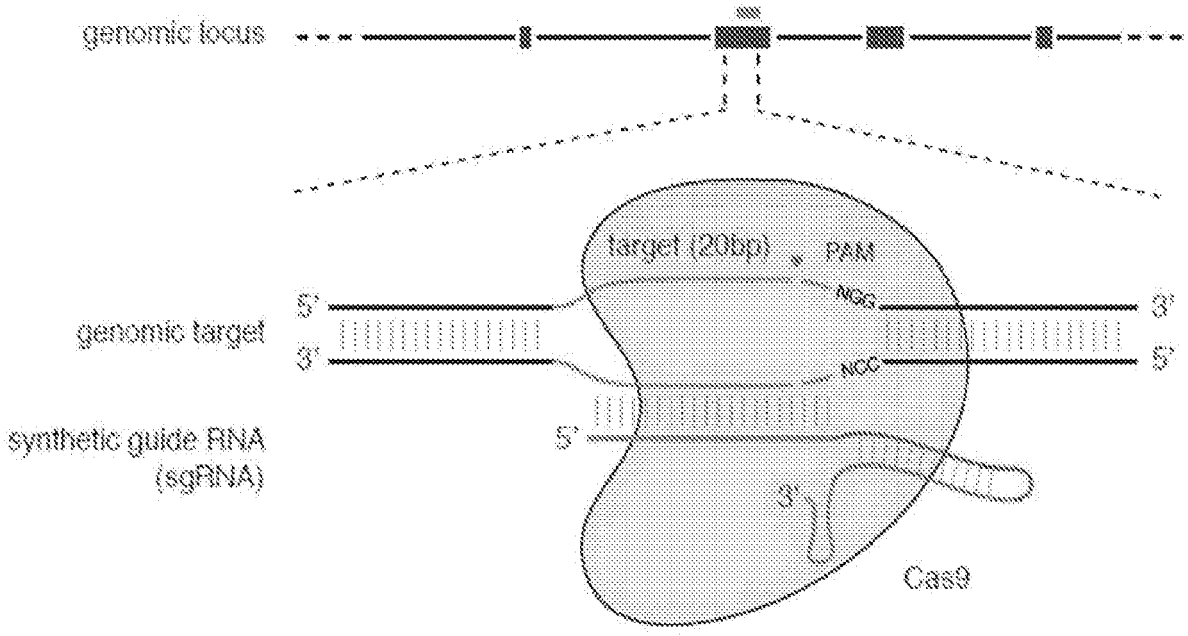

genomic locus
target (20bp)
PAM
NGG
NCC
5'
3'
genomic target
synthetic guide RNA
(sgRNA)
Cas9

FIG. 1

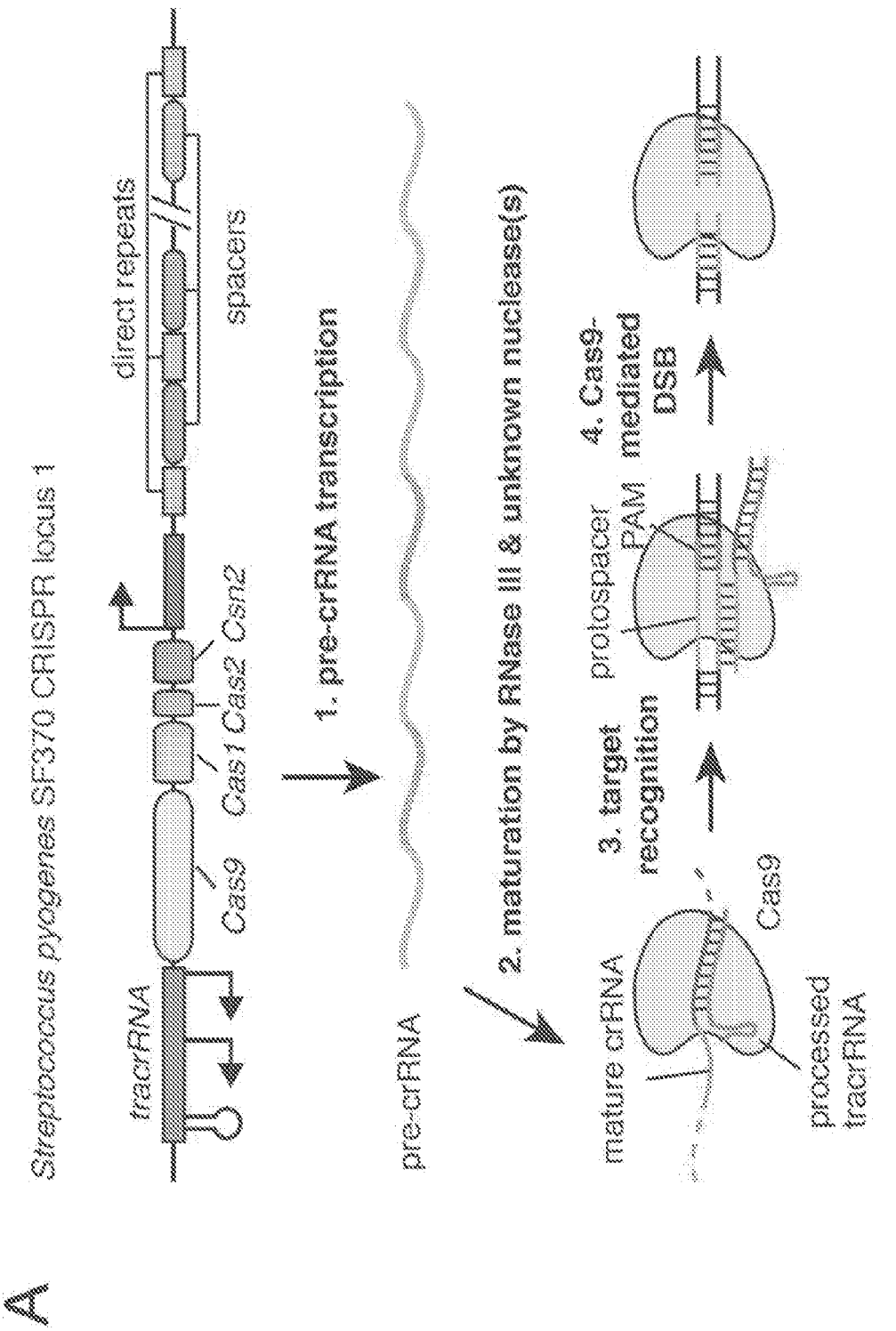

A
Streptococcus pyogenes SF370 CRISPR locus 1
direct repeats
spacers
tracrRNA
Cas9
Cas1
Cas2
Csn2
1. pre-crRNA transcription
pre-crRNA
2. maturation by RNase III & unknown nuclease(s)
mature crRNA
3. target recognition
protospacer
PAM
4. Cas9-mediated DSB
Cas9
processed tracrRNA

FIG. 2A

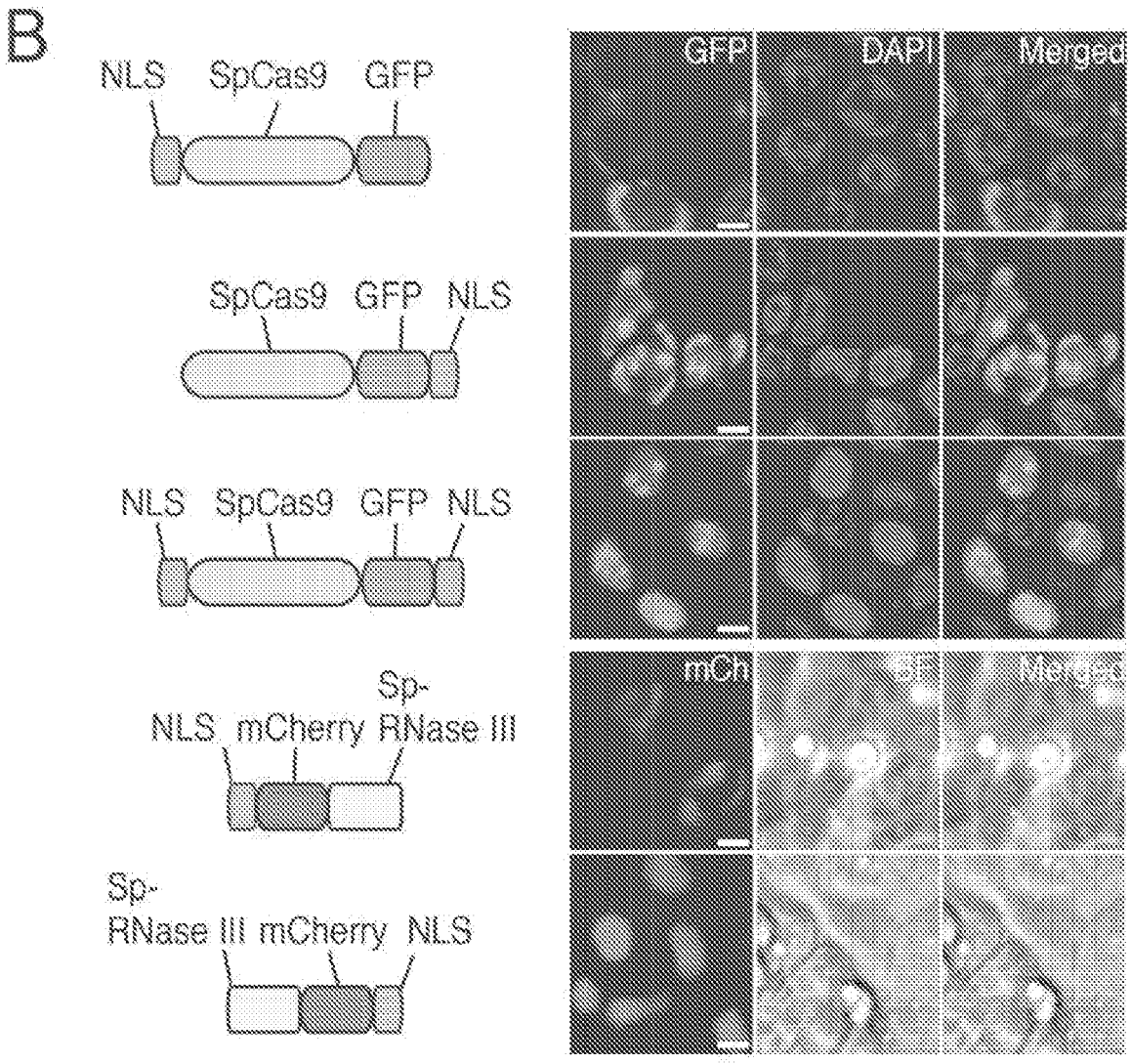

B
NLS SpCas9 GFP
SpCas9 GFP NLS
NLS SpCas9 GFP NLS
Sp-
NLS mCherry RNase III
Sp-
RNase III mCherry NLS
GFP
DAPI
Merged
mCh
GFP
Merged

FIG. 2B

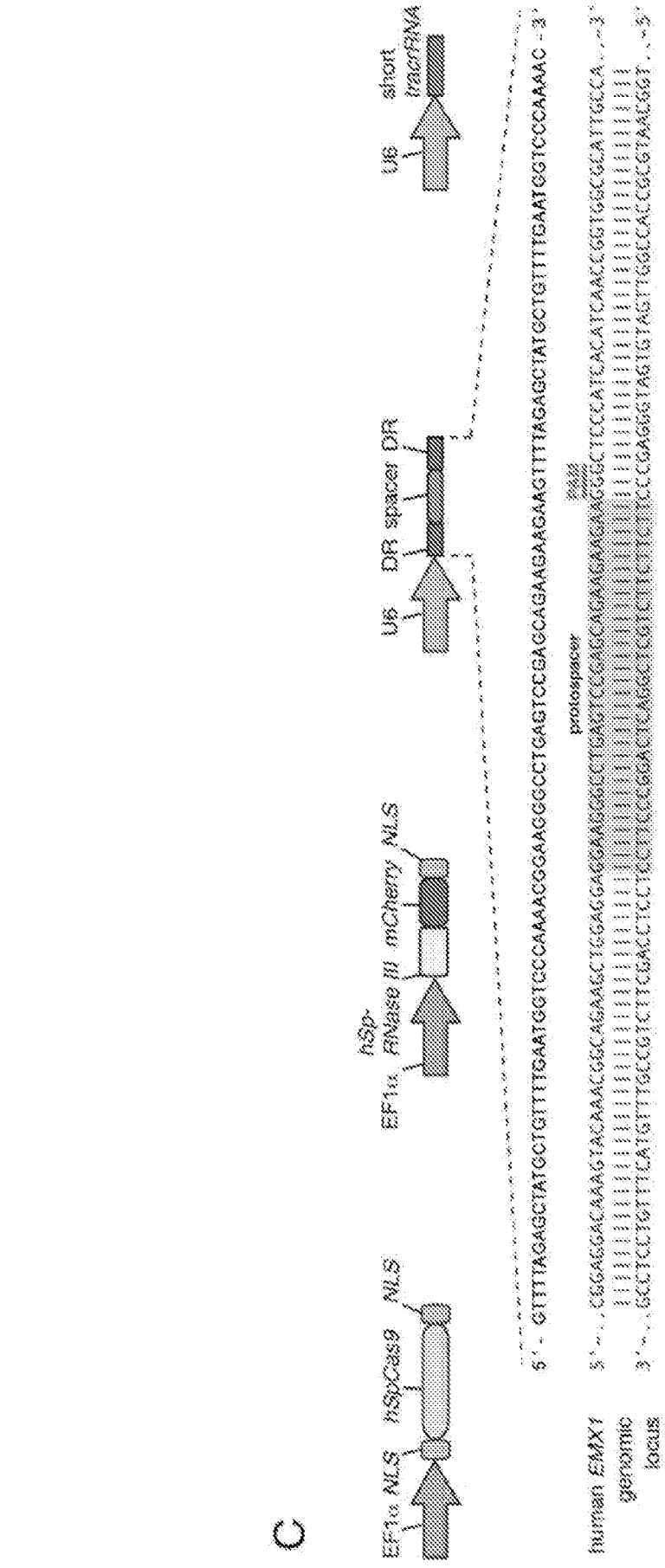

C
EF1α NLS hSpCas9 NLS
human EMX1 genomic locus
5' - ...GGGACACAAAGTACAAACGGCAGAAGCTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACATCAACCGGTGGCGCATTGCCA...-3'
3' - ...GCCTCCTGTTTCATGTTTGCCGTCTTCGACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCCCGAGGGTAGTGTAGTTGGCCACCGCGTAACGGT...-5'
protospacer
PAM
6' - GTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAAC - 3'
U6 DR spacer DR
U6 short tracrRNA
EF1α hSp. RNase III mCherry NLS

FIG. 2C

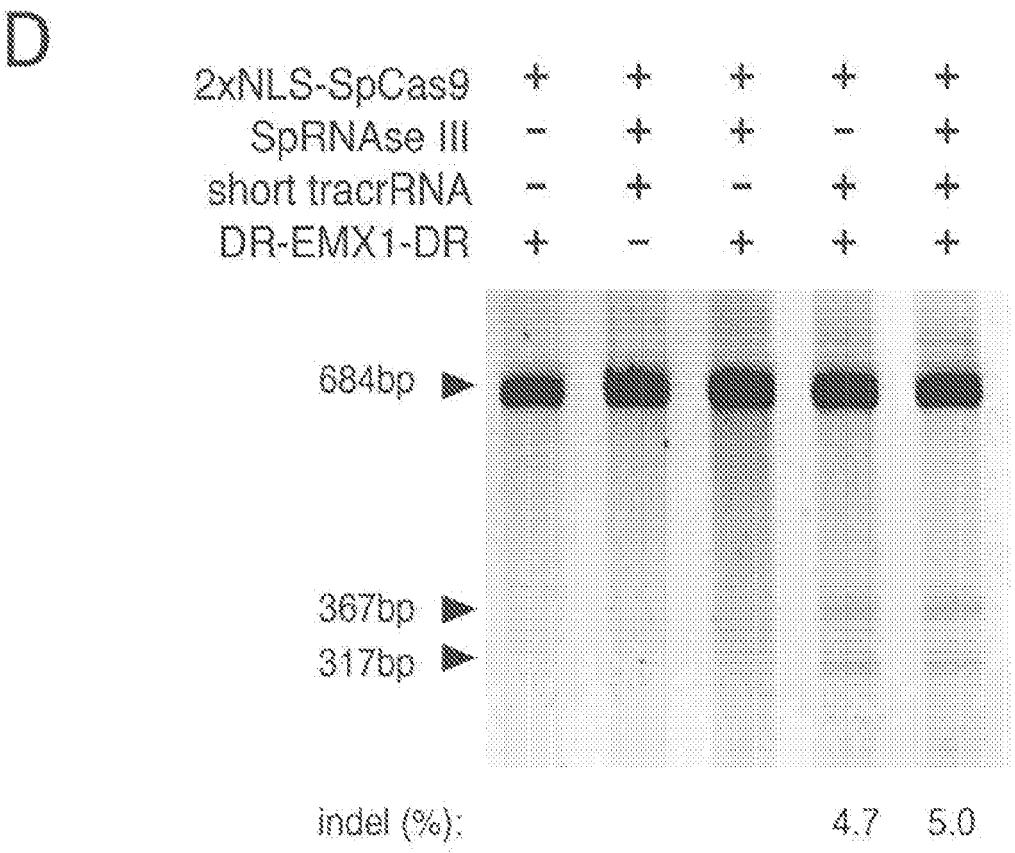

D
2xNLS-SpCas9 + + + + +
SpRNAse III - + + - +
short tracrRNA - + - + +
DR-EMX1-DR + - + + +
684bp
367bp
317bp
indel (%): 4.7 5.0

FIG. 2D

E
PAM
Target locus 5' - . . AGCTGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGGGCTCCCAC . . - 3'
3' - . . TCGACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCCCGAGGGTG . . - 5'
crRNA
5' - GAGUCCGAGCAGAAGAAGUUUUAGAGC . . - 3'
indel
AGCTGGAGGAAGGGCCTGAGTCCGAGCAGAAGA--GAAGGGCTCCCAT
F
human EMX1 protospacer target (mutation in 5 of 43 sequenced clones = 11.6%)
WT 5' - . . CTGGAGGAAGGGCCTGAGTCCGAGCAGAAG AAGAAGGGCTCCCATCACAT . . - 3'
Δ1 CTGGAGGAAGGGCCTGAGTCCGAGCAGAAG---AGAAGGGCTCCCATCACAT
+1 CTGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATCACAT
Δ3 CTGGAGGAAGGGCCTGAGTCCGAGCAGAAG----AAGGGCTCCCATCACAT
m1, Δ6 CTGGAGGAAGGGCCTGAGCCCGAGCAGAAG----------GGCTCCCATCACAT

FIG. 2E-F

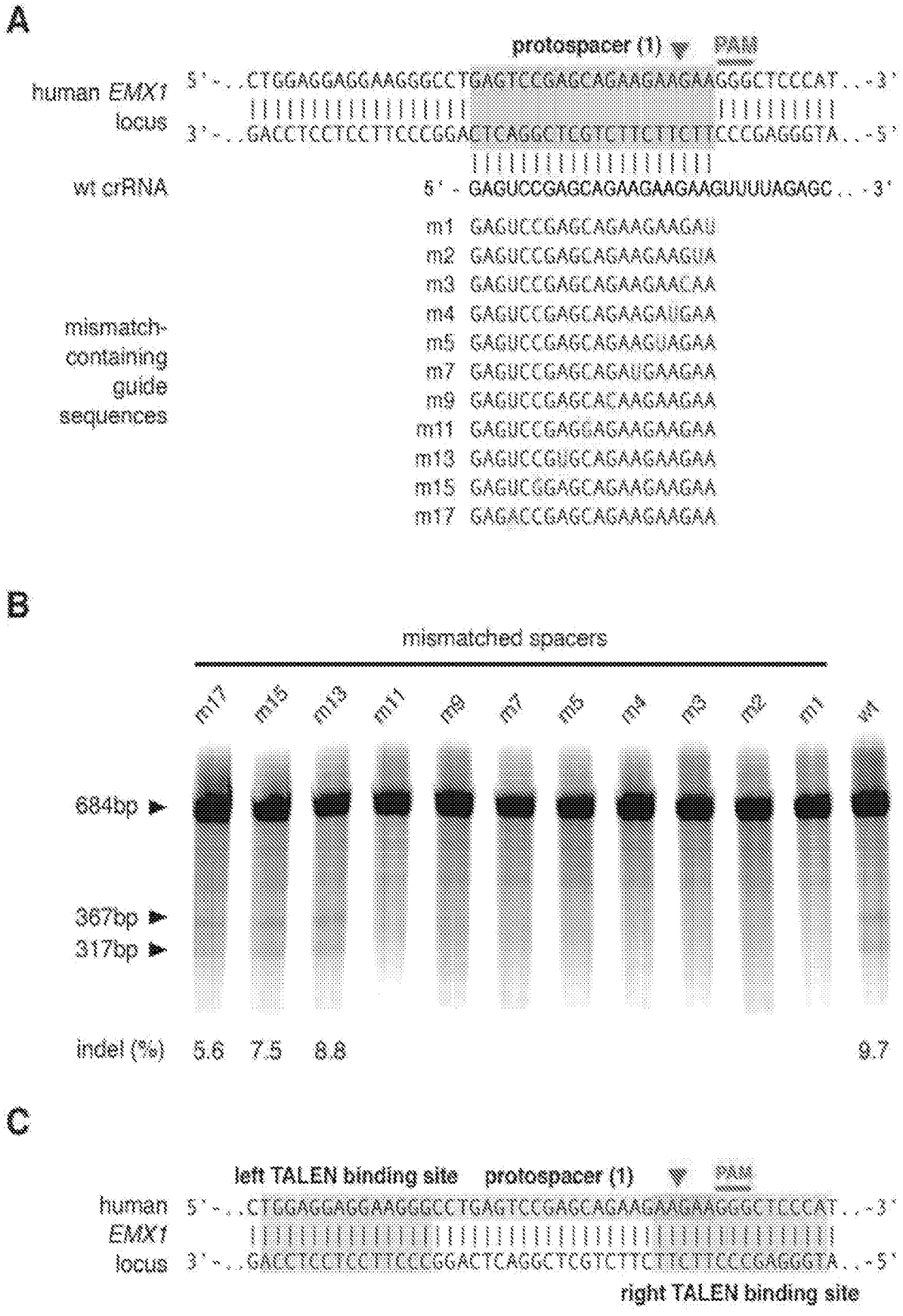

A
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
wt crRNA
5'-GAGUCCGAGCAGAAGAAGAAGUUUUAGAGC..-3'
mismatch-containing guide sequences
m1 GAGUCCGAGCAGAAGAAGAU
m2 GAGUCCGAGCAGAAGAAGUA
m3 GAGUCCGAGCAGAAGAACAA
m4 GAGUCCGAGCAGAAGAUGAA
m5 GAGUCCGAGCAGAAGUAGAA
m7 GAGUCCGAGCAGAUGAAGAA
m9 GAGUCCGAGCACAAGAAGAA
m11 GAGUCCGAGCAGAAGAAGAA
m13 GAGUCCGUGCAGAAGAAGAA
m15 GAGUCCGAGCAGAAGAAGAA
m17 GAGACCGAGCAGAAGAAGAA
B
mismatched spacers
m17
m15
m13
m11
m9
m7
m5
m4
m3
m2
m1
wt
684bp
367bp
317bp
indel (%)
5.6
7.5
8.8
9.7
C
left TALEN binding site
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
right TALEN binding site

FIG. 3A-C

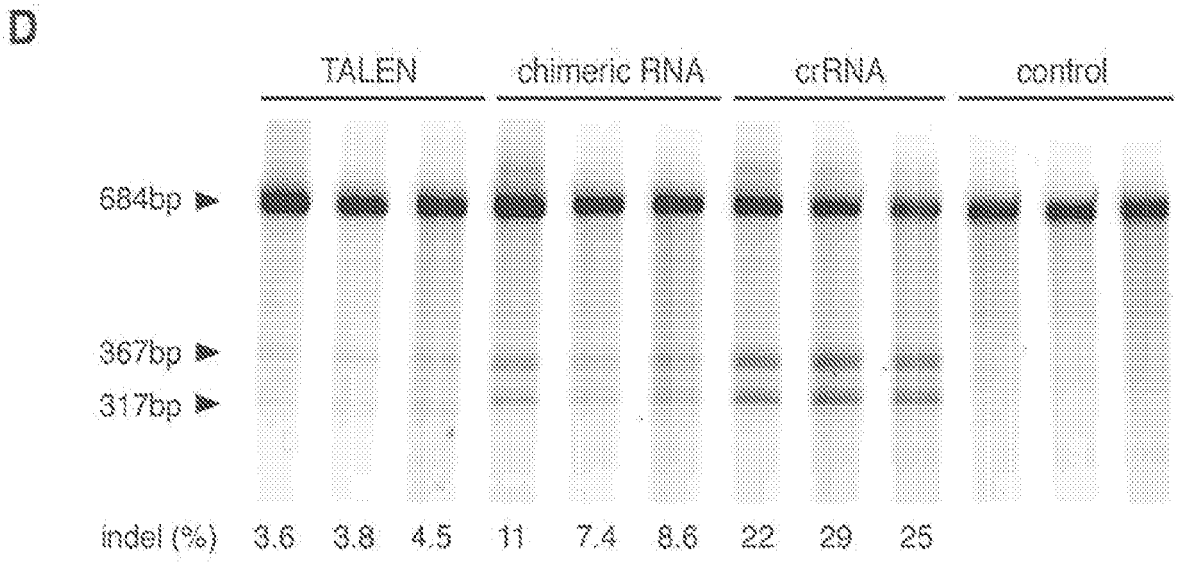

D
TALEN
chimeric RNA
crRNA
control
684bp
367bp
317bp
indel (%)
3.6
3.8
4.5
11
7.4
8.6
22
29
25

FIG. 3D

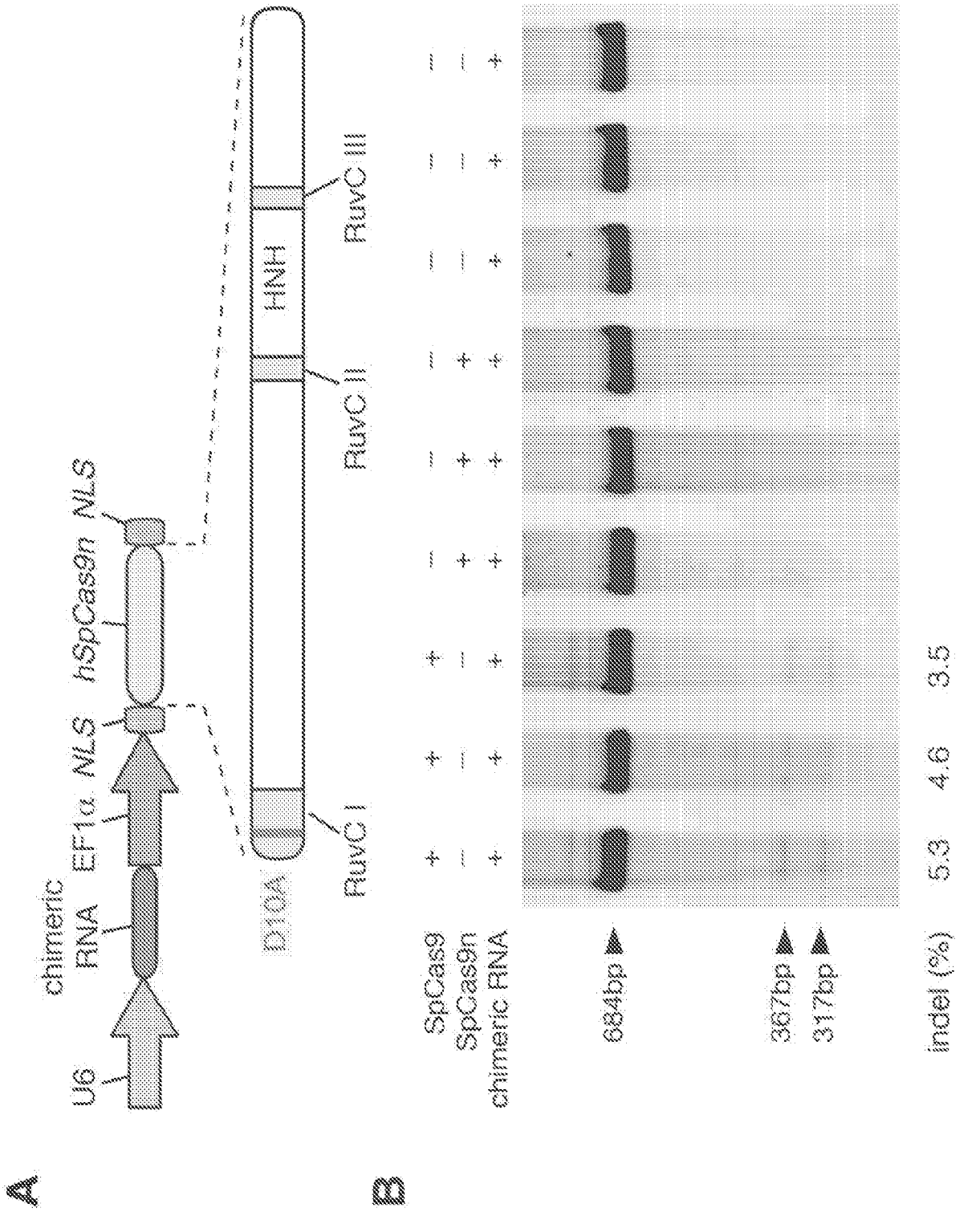

A
U6
chimeric RNA
EF1α
NLS
hSpCas9n
NLS
D10A
RuvC I
RuvC II
HNH
RuvC III
B
SpCas9
SpCas9n
chimeric RNA
684bp
367bp
317bp
indel (%) 5.3 4.6 3.5

FIG. 4A-B

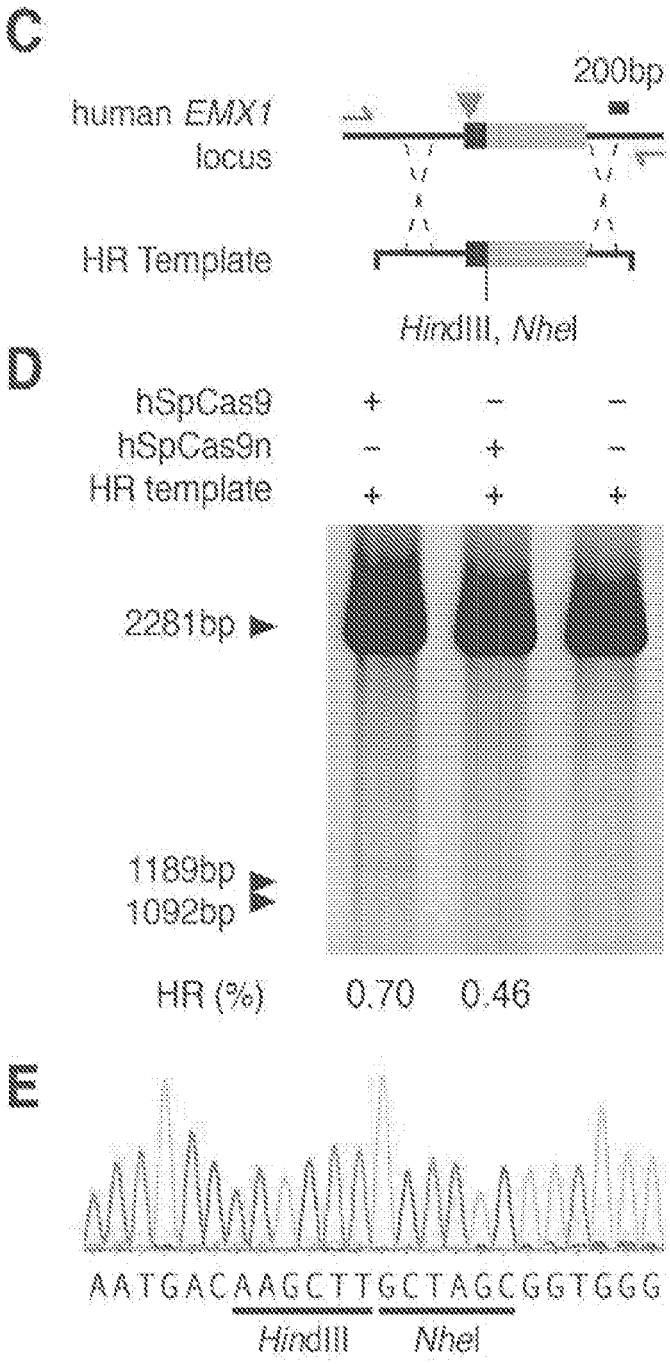

C
200bp
human EMX1 locus
HR Template
HindIII, NheI
D
hSpCas9
hSpCas9n
HR template
2281bp
1189bp
1092bp
HR (%)
0.70
0.46
E
AATGACAAGCTTGCTAGCGGTGGG
HindIII
NheI

FIG. 4C-E

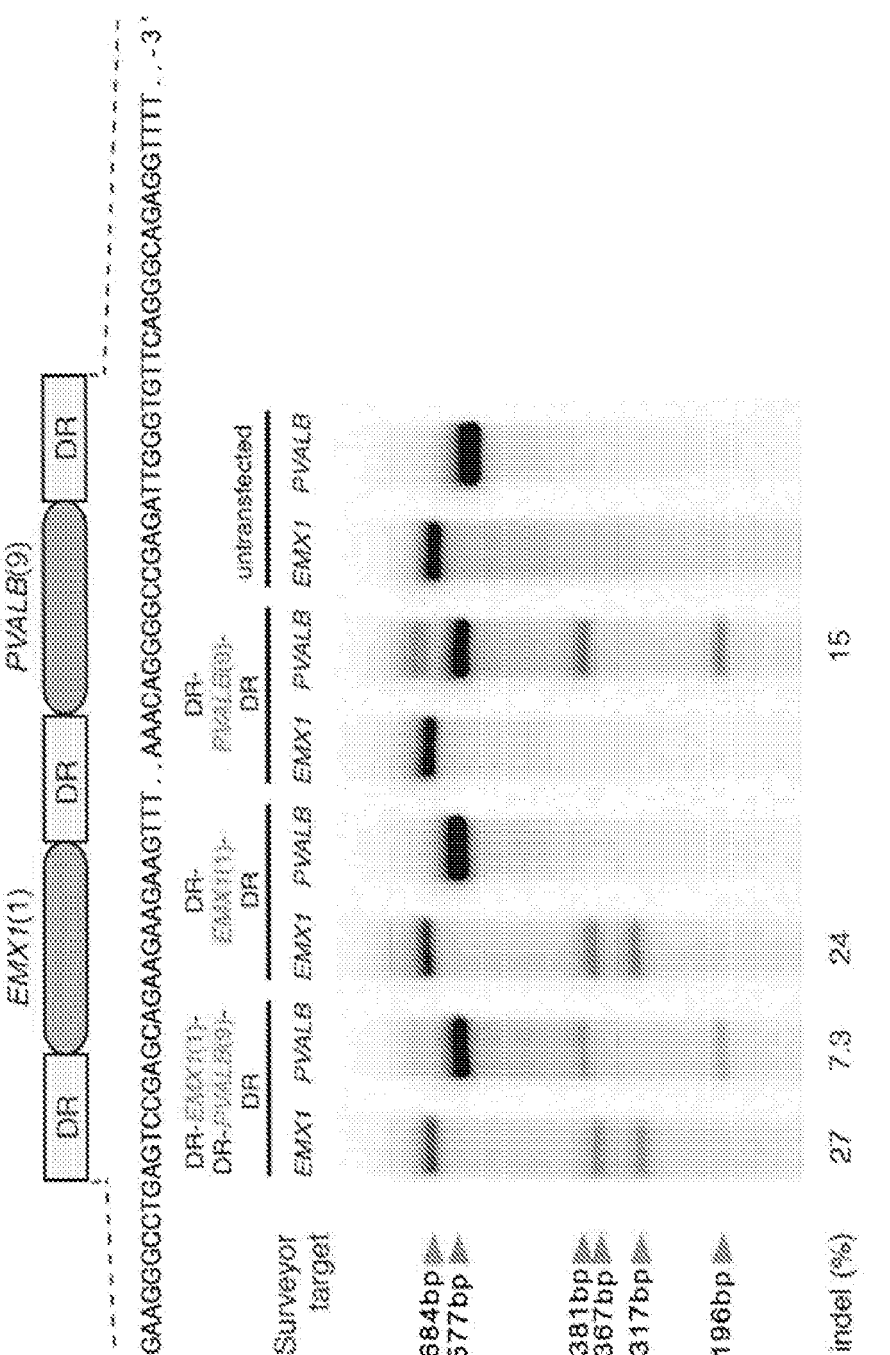

F
crRNA array design
EMX1(1)
PVALB(9)
DR
DR
DR
DR
5' - . . AAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTT . . AAACAGGGGCCGAGATTGGGTGTTCAGGGCAGAGGTTTT . . -3'
DR-EMX1(1)-DR-PVALB(9)-DR
DR-EMX1(1)-DR
DR-PVALB(9)-DR
untransfected
Surveyor target
EMX1
PVALB
EMX1
PVALB
EMX1
PVALB
EMX1
PVALB
684bp
577bp
381bp
367bp
317bp
196bp
indel (%)
27
7.3
24
15

FIG. 4F

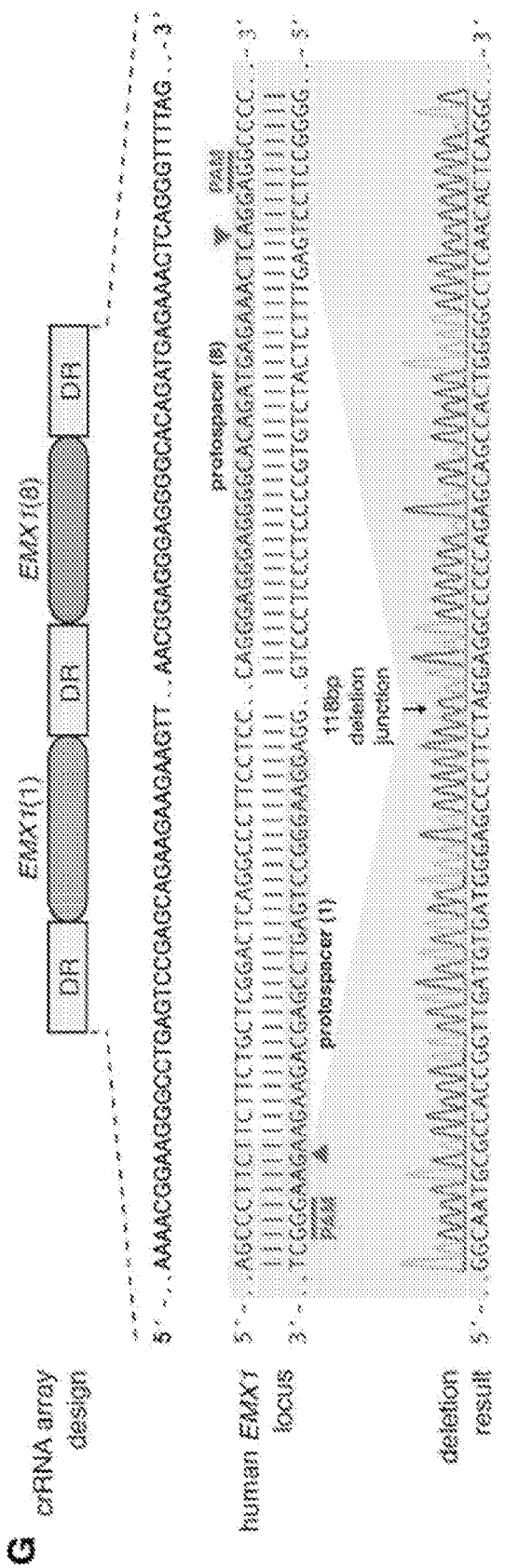

G
crRNA array design
DR
DR
DR
EMX1(1)
EMX1(8)
5' - - - AAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTT - - - AACGGAGGGAGGGCACAGATGAGAAACTCAGGGTTTTAG - - - 3'
human EMX1 locus
5' - - - AGCCCTTCTTCTTCTGCTCGGACTCAGGCCCTTCCTCC - - - CAGGGAGGGAGGGCACAGATGAGAAACTCAGGAGGCCCC - - - 3'
3' - - - TCGGGAAGAAGACGAGCCTGAGTCCGGGAAGGAGG - - - GTCCCTCCCTCCCCGTGTCTACTCTTTGAGTCCTCCGGGG - - - 5'
protospacer (1)
PAM
protospacer (8)
PAM
118bp deletion junction
deletion result
5' - - - GGCAATGCGCCACCGGTTGATGTGATGGGAGCCCTTCTAGGAGGCCCCCAGAGCCAGCCACTGGGGCCCTCAACACTCAGGCC - - - 3'

FIG. 4G

Cas9	target species	gene	protospacer ID	protospacer sequence (5' to 3')	PAM	strand	cell line tested	% indel (pre-crRNA + tracrRNA)	% indel (chimeric RNA)
S. pyogenes SF370 type II CRISPR	*Homo sapiens*	*EMX1*	1	GGAGGGCCTGAGTCCGAGCAGAAGAAA	GGG	+	293FT	20 ± 1.3	6.7 ± 0.62
		EMX1	2	CATTGAGGTGACATCGATGTCCTCCCAT	TGG	–	293FT	2.1 ± 0.31	N.D.
		EMX1	3	GGACATCGATGTCACCTCCAATGACTAGGG	TGG	+	293FT	14 ± 1.1	N.D.
		EMX1	4	CATCGATGTCCTCCCCATTGGCCTGCTTCG	TGG	–	293FT	11 ± 1.7	N.D.
		EMX1	5	TTCGTGCAATGCGCCAACCGGTTGATGTCA	TGG	–	293FT	4.3 ± 0.46	2.1 ± 0.31
		EMX1	6	TCGTGCAATGCGCCAACCGGTTGATGTCAT	GGG	–	293FT	4.0 ± 0.60	0.41 ± 0.25
		EMX1	7	TCCAGCTTCTGCGTTTGTACTTTGTCCTC	CGG	–	293FT	1.6 ± 0.12	N.D.
		EMX1	8	GGAGGAGGGCACAGATGAGAACTCAGG	AGG	–	293FT	7.8 ± 0.83	2.3 ± 1.2
	Homo sapiens	*PVALB*	9	AGGGCCCACATTGGGTGTTCAGGCAGAG	AGG	+	293FT	21 ± 2.6	6.5 ± 0.32
		PVALB	10	ATGCAGGAGGTGCCGAGAGGGCCGAGAT	TGG	+	293FT	N.D.	N.D.
		PVALB	11	GGTGCCGAGAGGGCCGAGATTGGGTGTTC	AGG	+	293FT	N.D.	N.D.
	Mus musculus	*Th*	12	CAAGCACTGAGTGCCATTAGCTAAATGCAT	AGG	–	Neuro2A	27 ± 4.3	4.1 ± 2.2
		Th	13	AATGCATAGGGTACCACCCACAGGTGCCAG	GGG	–	Neuro2A	4.8 ± 1.2	N.D.
		Th	14	ACACACATGGCAAAGCCCTCTGGGCCAGGAA	AGG	+	Neuro2A	11.3 ± 1.3	N.D.
S. thermophilus LMD-9 CRISPR1	*Homo sapiens*	*EMX1*	15	GGAGGAGGTAGTAATACAGAAACACAGAGAA	GTAGAAT	–	293FT	14 ± 0.88	N.T.
		EMX1	16	AGAATGTAGAGGAGTCACAGAAACTCAGCA	CTAGAAA	–	293FT	7.8 ± 0.77	N.T.

FIG. 5

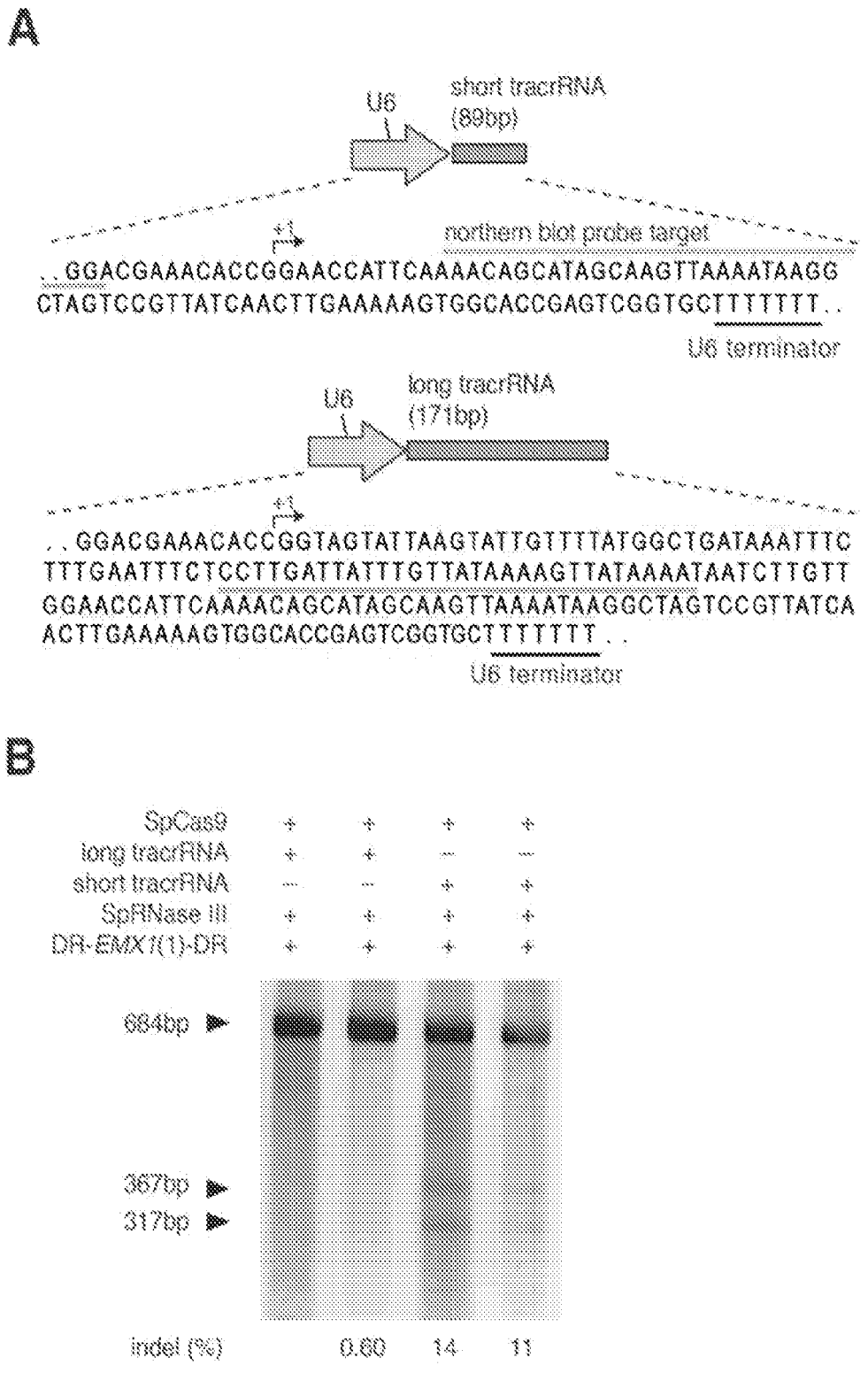

A
U6
short tracrRNA
(89bp)
+1
northern blot probe target
..GGACGAAACACCGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
U6
long tracrRNA
(171bp)
+1
..GGACGAAACACCGGTAGTATTAAGTATTGTTTTATGGCTGATAAATTTC
TTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTT
GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
B
SpCas9 + + + +
long tracrRNA + + – –
short tracrRNA – – + +
SpRNase III + + + +
DR-EMX1(1)-DR + + + +
684bp
367bp
317bp
indel (%) 0.60 14 11

FIG. 6A-B

C

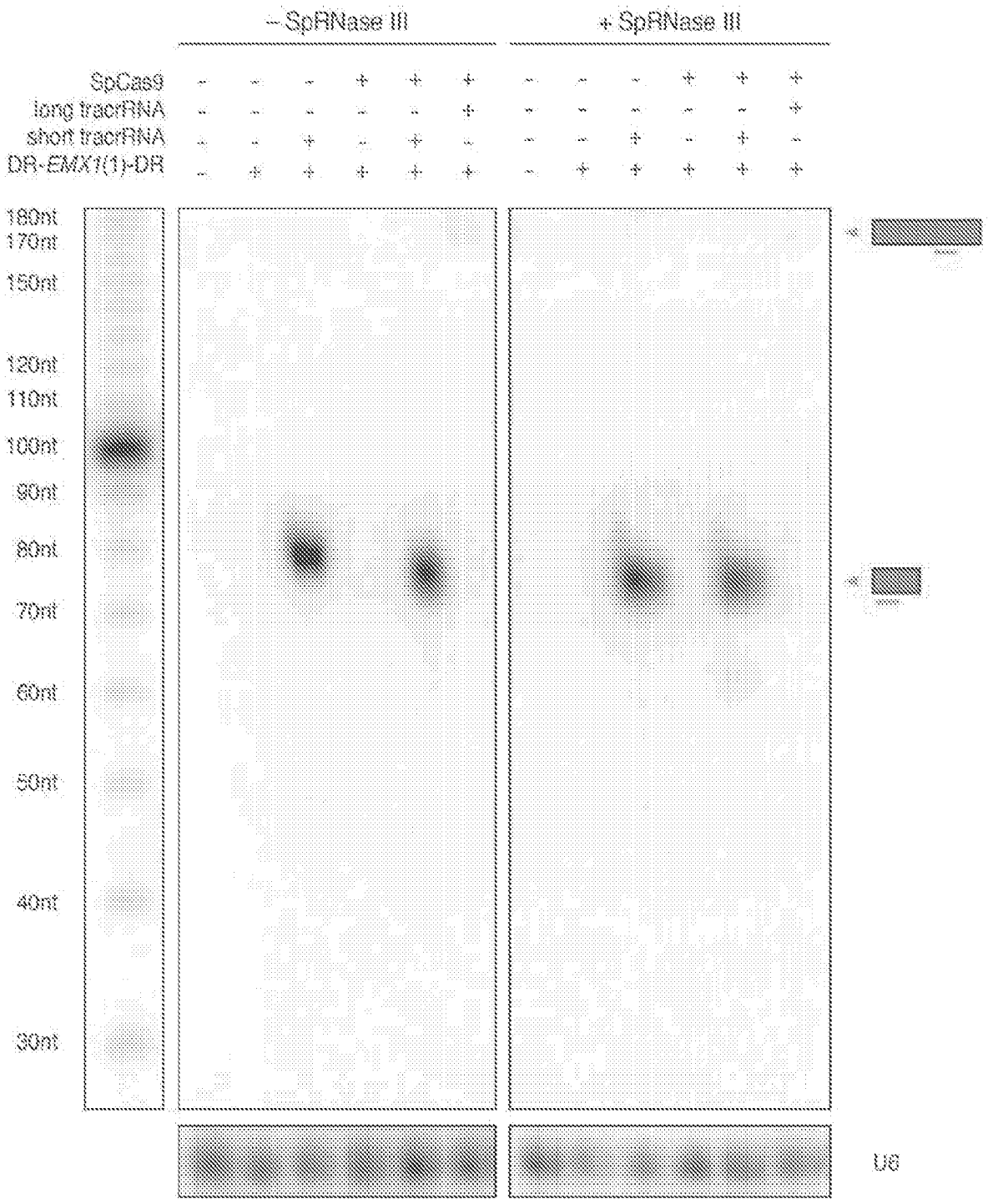

−SpRNase III
+SpRNase III
SpCas9
long tracrRNA
short tracrRNA
DR-EMX1(1)-DR
180nt
170nt
150nt
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 6C

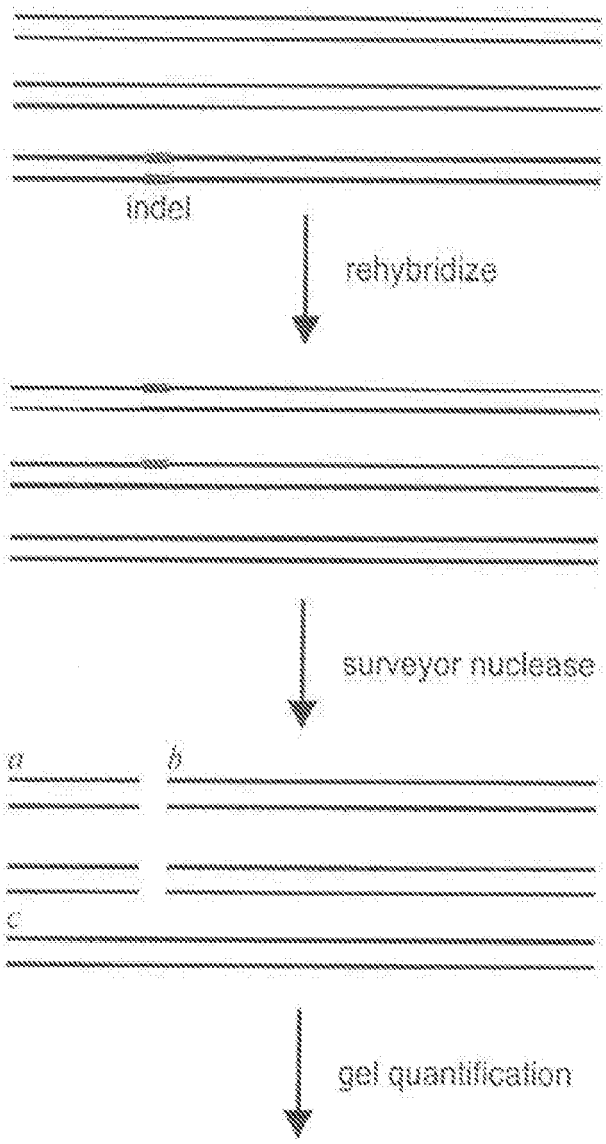

indel
rehybridize
surveyor nuclease
a
b
c
gel quantification
% indel = (1 - √1 - (a + b)/(a + b + c)) * 100

FIG. 7

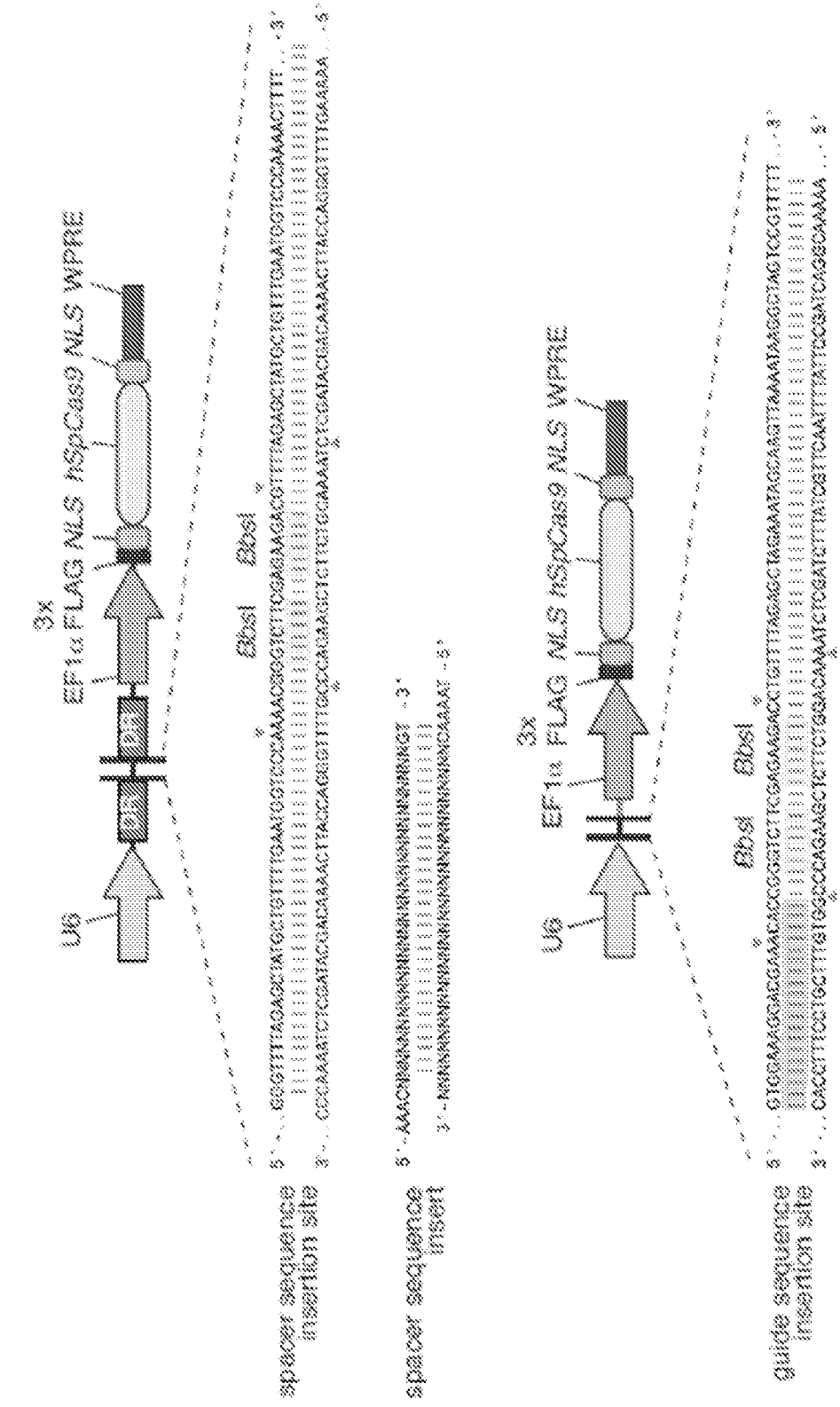
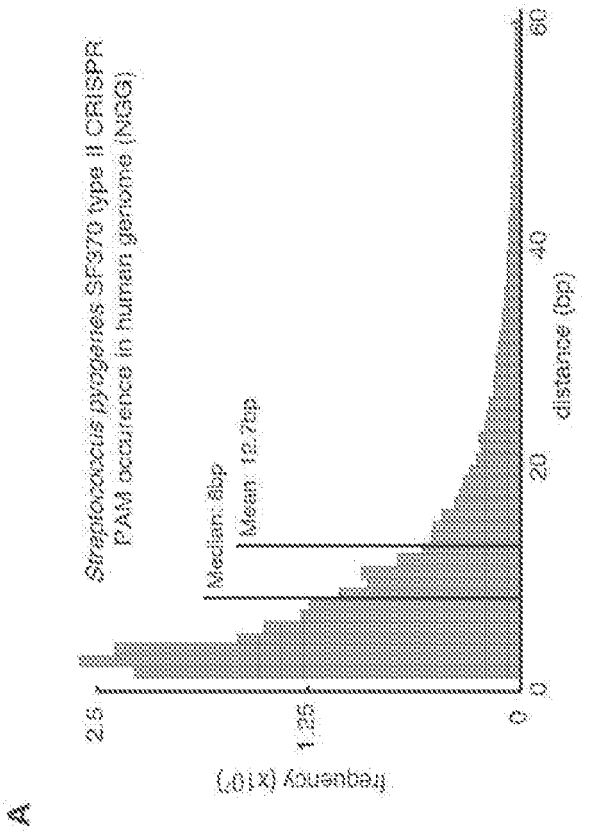
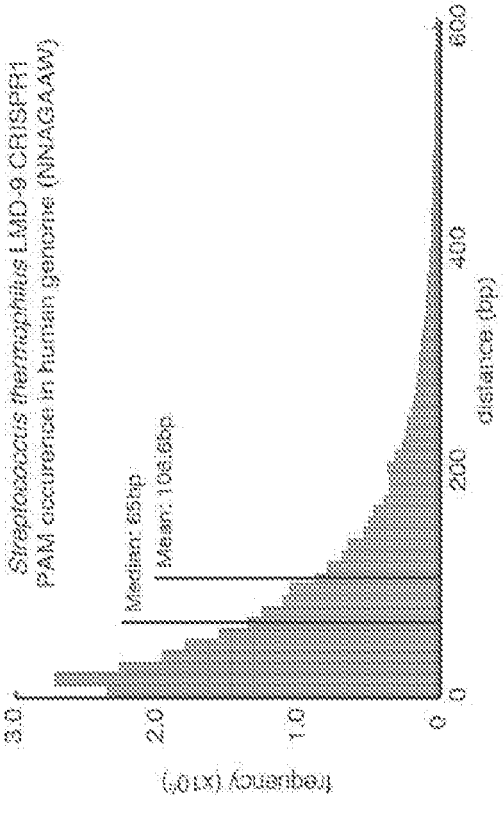

A
U6
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
BbsI
BbsI
spacer sequence insertion site
spacer sequence insert
B
U6
3x
EF1α FLAG NLS hSpCas9 NLS WPRE
BbsI
BbsI
guide sequence insertion site
guide sequence insert

FIG. 8A-B





C

	NGG		NNAGAAW	
Chr	median	mean	median	mean
1	7	12.8	67	115.8
2	8	12.7	64	100.8
3	8	13.0	63	98.5
4	9	14.0	61	94.5
5	8	13.1	63	97.9
6	8	13.1	63	98.5
7	8	12.4	64	102.9
8	8	12.8	64	100.9
9	7	13.9	65	120.5
10	7	12.1	66	107.0
11	7	12.0	65	105.8
12	8	12.4	65	103.5
13	8	13.6	62	94.6
14	8	12.0	65	101.5
15	7	11.5	68	107.7
16	7	11.7	74	136.8
17	6	10.3	76	127.9
18	8	13.4	63	101.8
19	6	9.4	82	145.4
20	7	11.1	72	121.8
21	7	13.4	64	111.4
22	6	9.2	85	140.3
X	8	13.2	63	99.0
Y	8	29.2	62	223.7

FIG. 9A-C

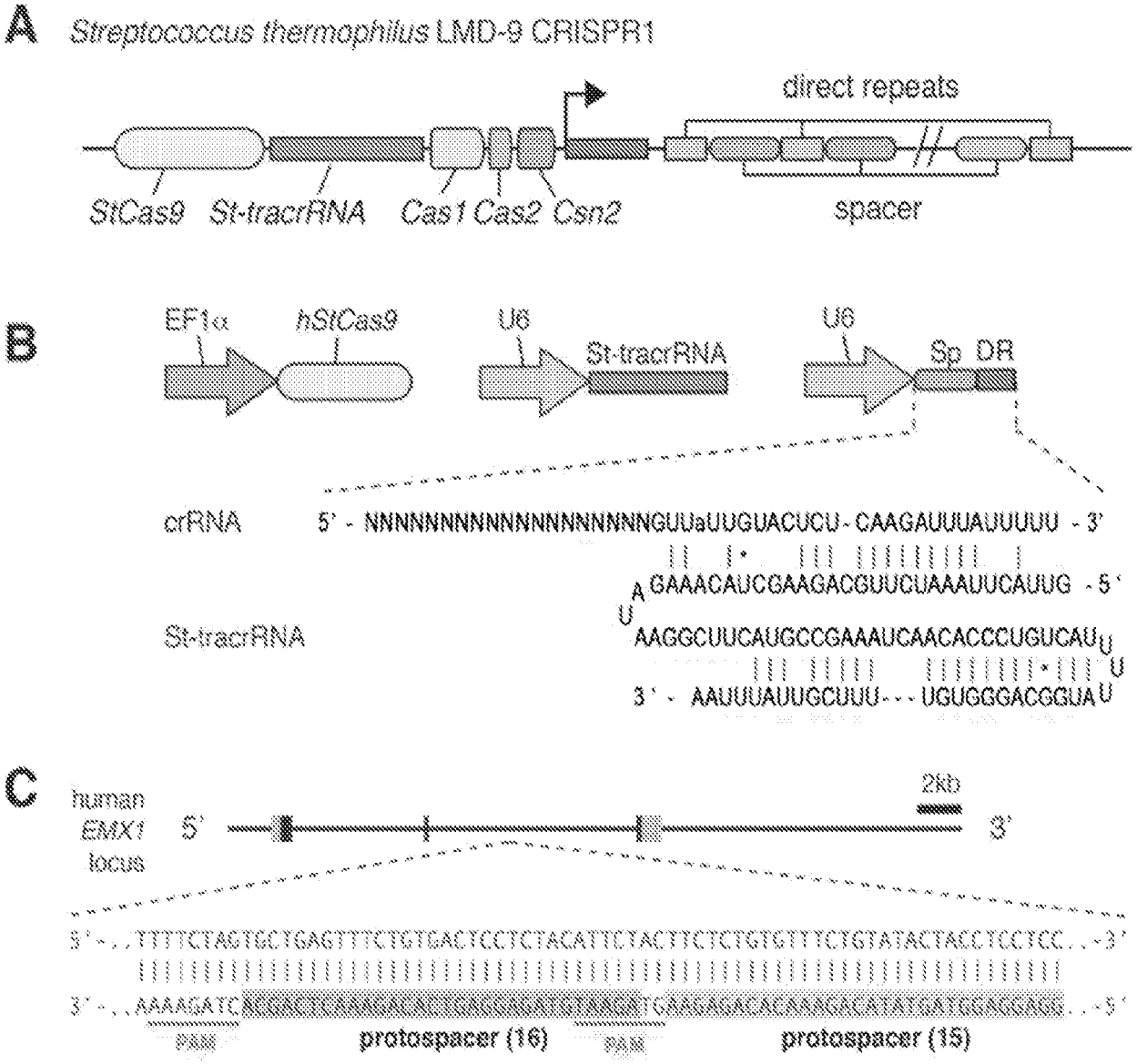

A
Streptococcus thermophilus LMD-9 CRISPR1
direct repeats
StCas9
St-tracrRNA
Cas1
Cas2
Csn2
spacer
B
EF1α
hStCas9
U6
St-tracrRNA
U6
Sp
DR
crRNA
5' - NNNNNNNNNNNNNNNNNNNGUUaUUGUACUCU - CAAGAUUUAUUUUU - 3'
A GAAACAUCGAAGACGUUCUAAAUUCAUUG - 5'
St-tracrRNA
U AAGGCUUCAUGCCGAAAUCAACACCCUGUCAU U
3' - AAUUUAUUGCUUU - - - UGUGGGACGGUA U
C
human
EMX1
locus
5'
2kb
3'
protospacer (16)
protospacer (15)

FIG. 10A-C

D

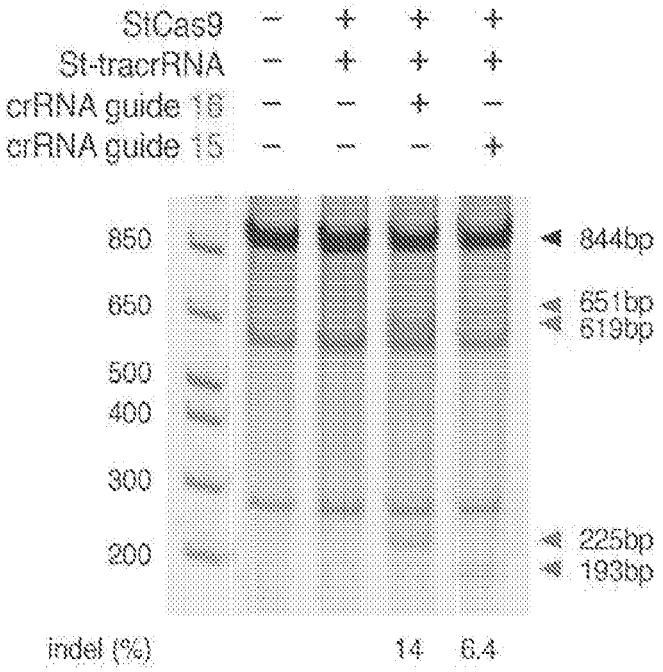

StCas9 – + + +
St-tracrRNA – + + +
crRNA guide 18 – – + –
crRNA guide 15 – – – +
850
650
500
400
300
200
844bp
651bp
619bp
225bp
193bp
indel (%)
14
6.4

FIG. 10D

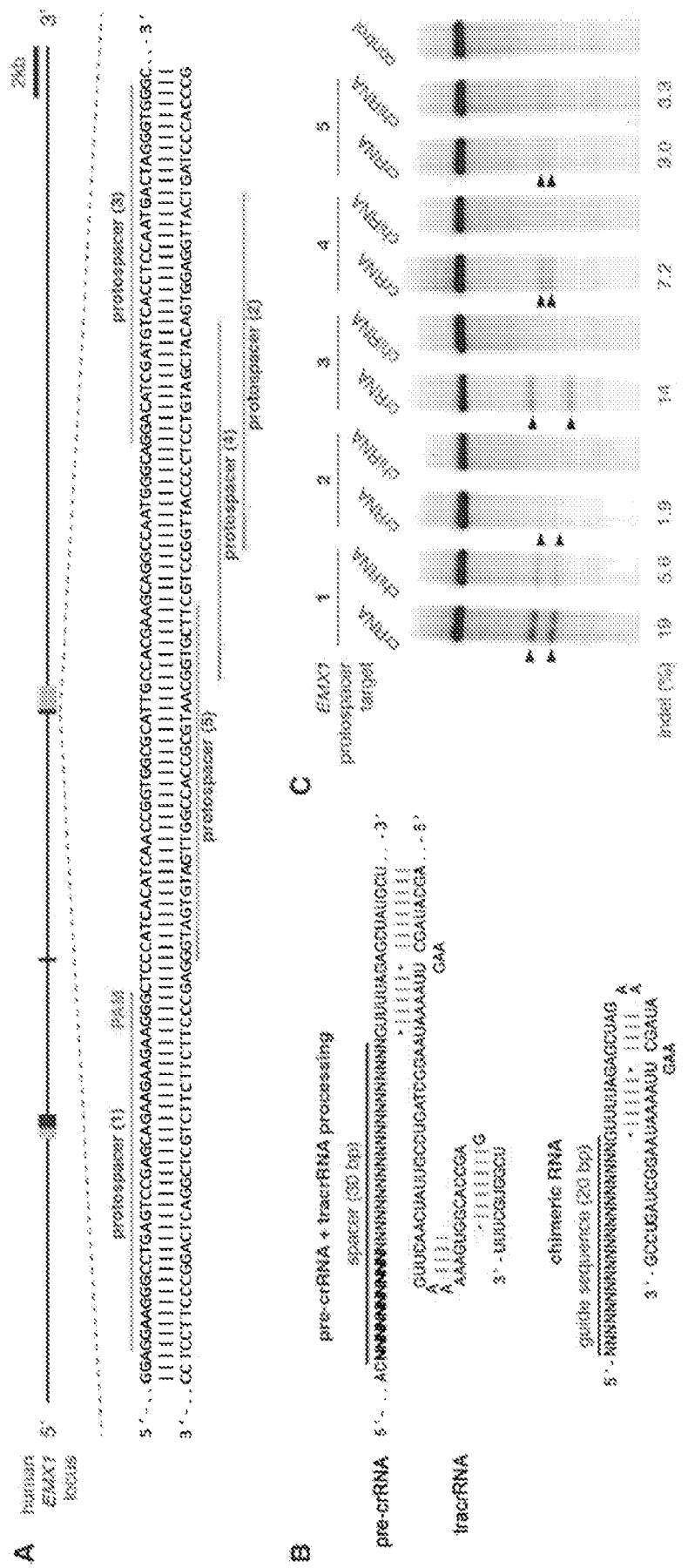

A
human EMX1 locus
5'
3'
2kb
protospacer (1)
PAM
protospacer (3)
protospacer (5)
protospacer (4)
protospacer (2)
B
pre-crRNA
tracrRNA
pre-crRNA + tracrRNA processing
spacer (20 bp)
chimeric RNA
guide sequence (20 bp)
C
EMX1 protospacer target
1
2
3
4
5
Indel (%)
19
5.8
1.9
1.4
7.2
3.0
6.3

FIG. 11A-C

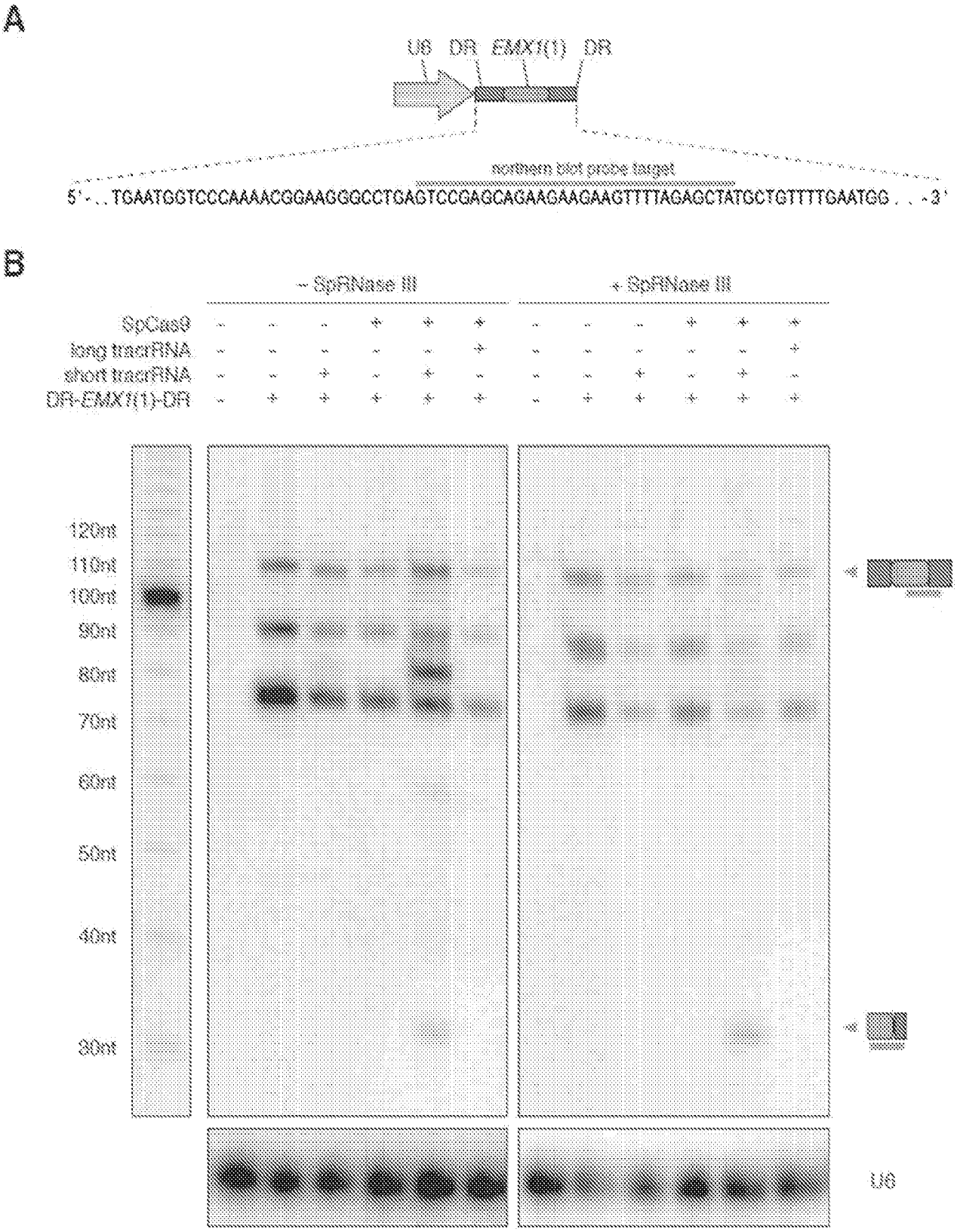

A
U6 DR EMX1(1) DR
northern blot probe target
5' . . . TGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTATGCTGTTTTTGAATGG . . . 3'
B
– SpRNase III
+ SpRNase III
SpCas9
long tracrRNA
short tracrRNA
DR-EMX1(1)-DR
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 12A-B

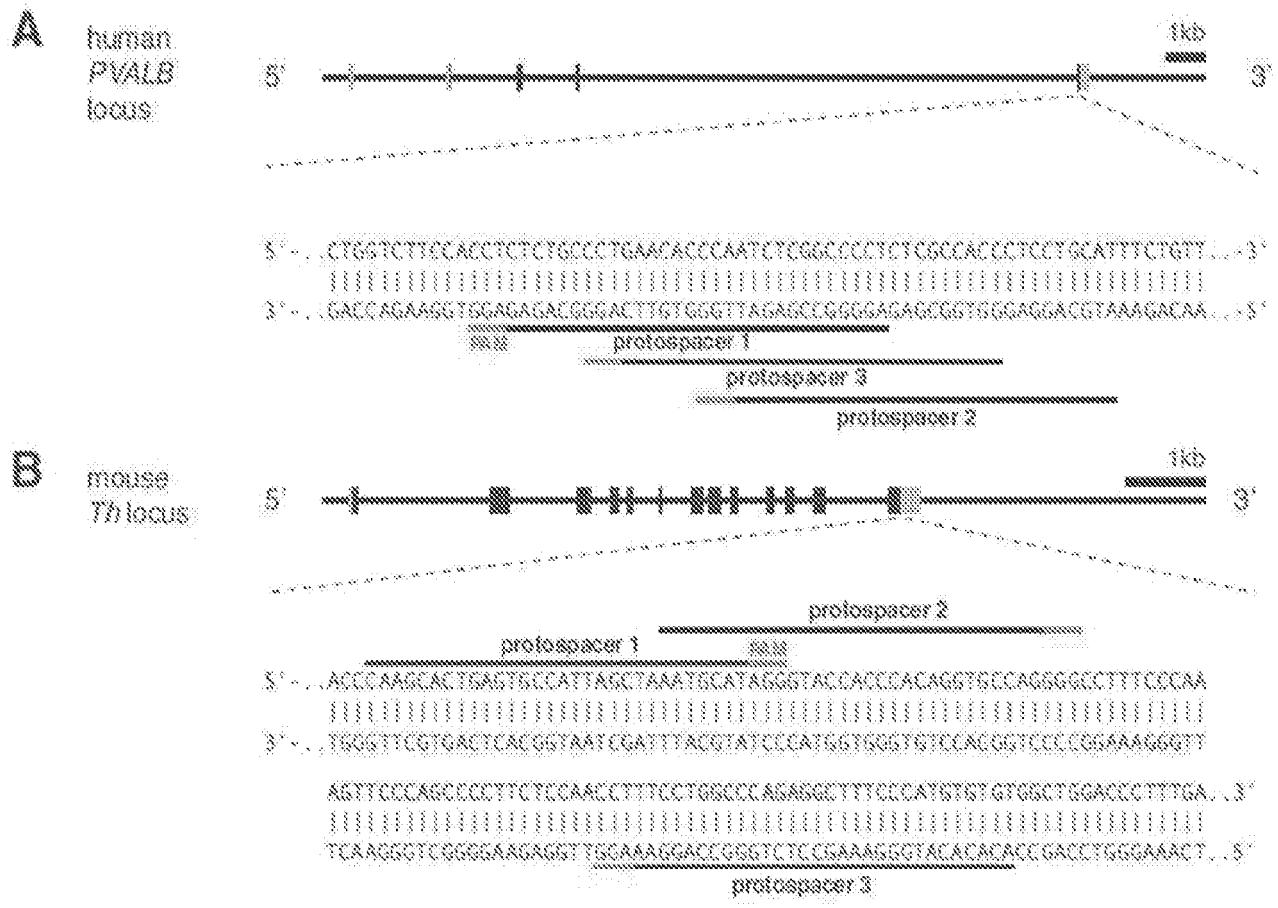

A
human
PVALB
locus
5'
1kb
3'
protospacer 1
protospacer 3
protospacer 2
B
mouse
Th locus
5'
1kb
3'
protospacer 2
protospacer 1
protospacer 3

FIG. 13A-B

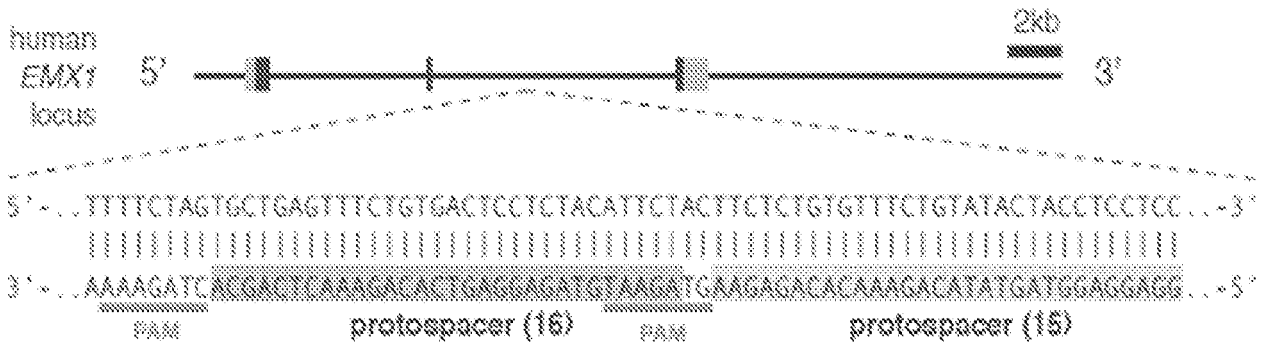

human
EMX1
locus
5'
2kb
3'
5'-..TTTTCTAGTGCTGAGTTTCTGTGACTCCTCTACATTCTACTTCTCTGTGTTTCTGTATACTACCTCCTCC..-3'
3'-..AAAAGATC
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 14

Primer name	Assay	Genomic Target	Primer sequence
Sp-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	AAAACCACCCTTCTCTCTGGC
Sp-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	GGAGATTGGAGACACGGAGAG
Sp-PVALB-F	SURVEYOR assay, sequencing	*PVALB*	CTGGAAAGCCAATGCCTGAC
Sp-PVALB-R	SURVEYOR assay, sequencing	*PVALB*	GGCAGCAAACTCCTTGTCCT
Sp-Th-F	SURVEYOR assay, sequencing	*Th*	GTGCTTTGCAGAGGCCTACC
Sp-Th-R	SURVEYOR assay, sequencing	*Th*	CCTGGAGCGCATGCAGTAGT
St-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	ACCTTCTGTGTTTCCACCATTC
St-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	TTGGGGAGTGCACAGACTTC
Sp-EMX1-RFLP-F	RFLP, sequencing	*EMX1*	GGCTCCCTGGGTTCAAAGTA
Sp-EMX1-RFLP-R	RFLP, sequencing	*EMX1*	AGAGGGGTCTGGATGTCGTAA
Pb_EMX1_sp1	Northern Blot Probe	Not applicable	TAGCTCTAAAACTTCTTCTTCTGCTCGGAC
Pb_tracrRNA	Northern Blot Probe	Not applicable	CTAGCCTTATTTTAACTTGCTATGCTGTTT

FIG. 15

a

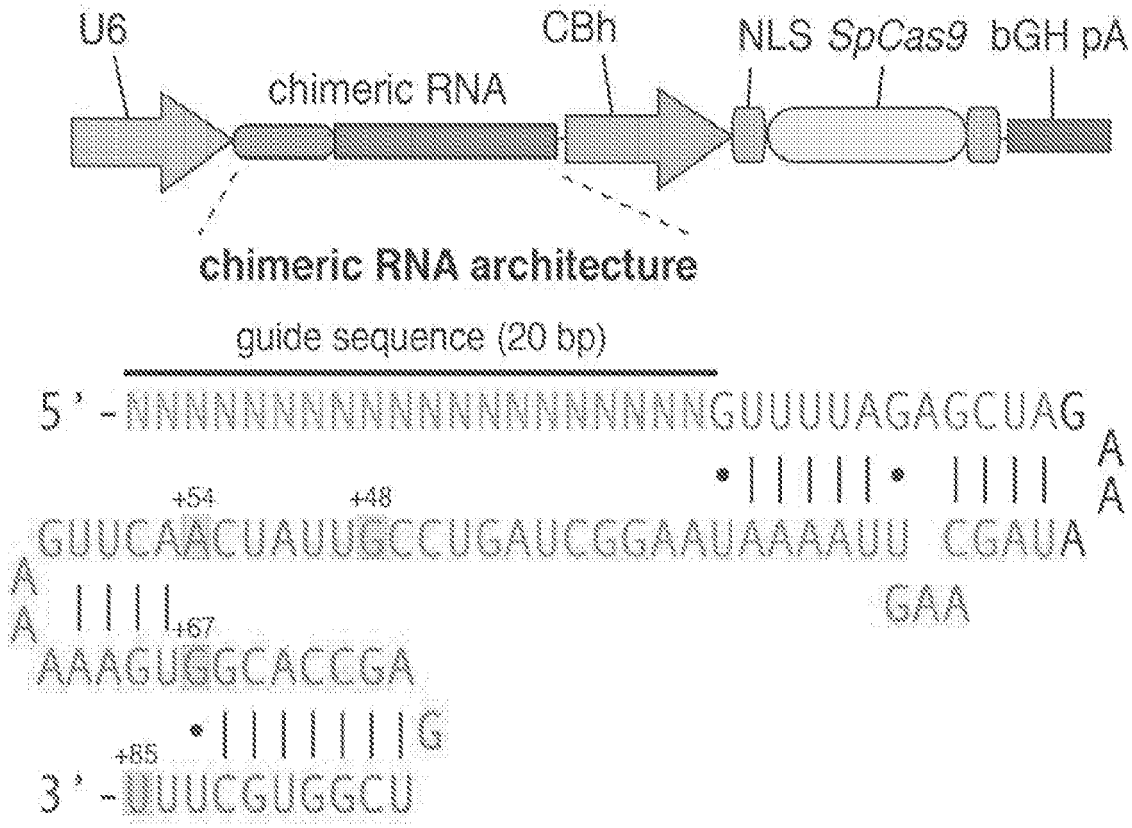

U6
chimeric RNA
CBh
NLS SpCas9 bGH pA
chimeric RNA architecture
guide sequence (20 bp)
5' - NNNNNNNNNNNNNNNNNNGUUUUAGAGCUAG
A
A
+54
+48
GUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAUA
A
A
GAA
+67
AAAGUGGCACCGA
G
+85
3' - UUUCGUGGCU

FIG. 16A

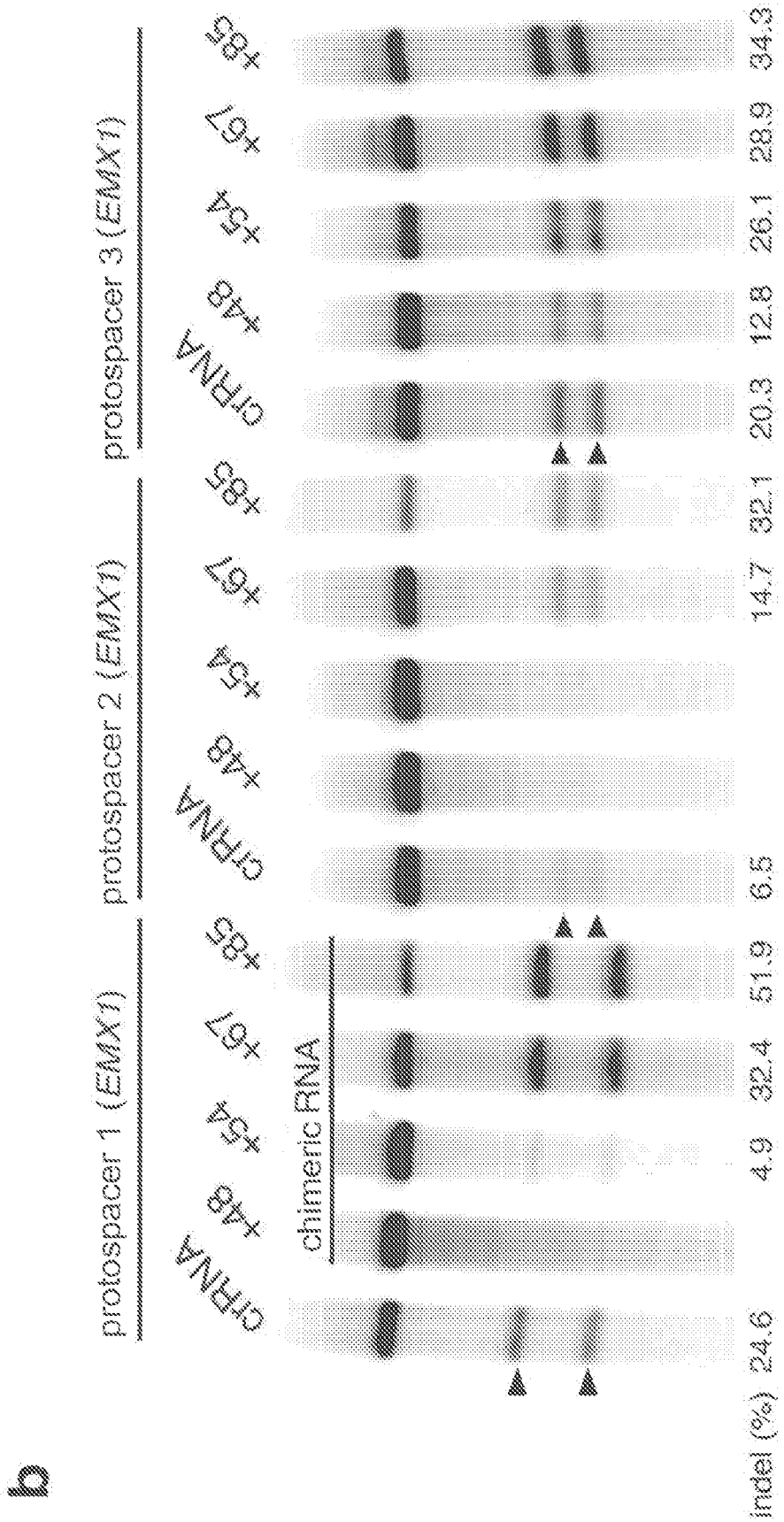

b
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
gRNA
chimeric RNA
indel (%)
24.6
4.9
32.4
51.9
6.5
14.7
32.1
20.3
12.8
26.1
28.9
34.3

FIG. 16B

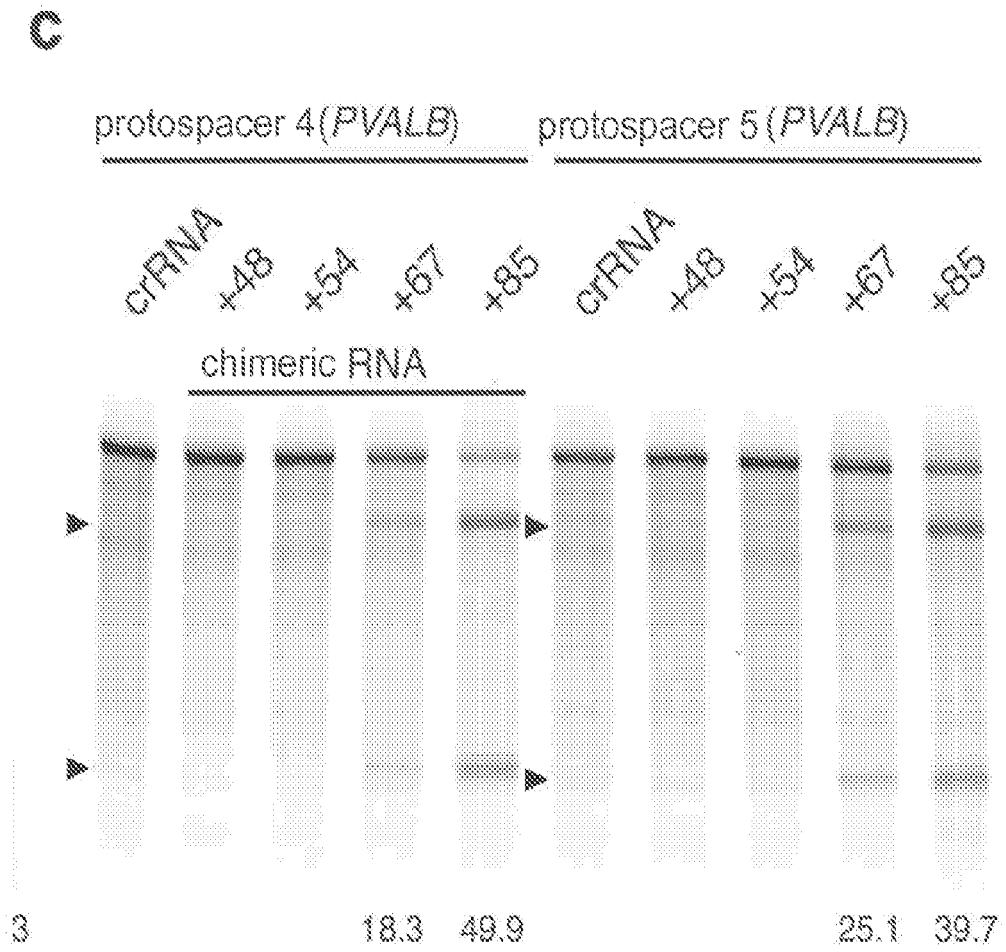
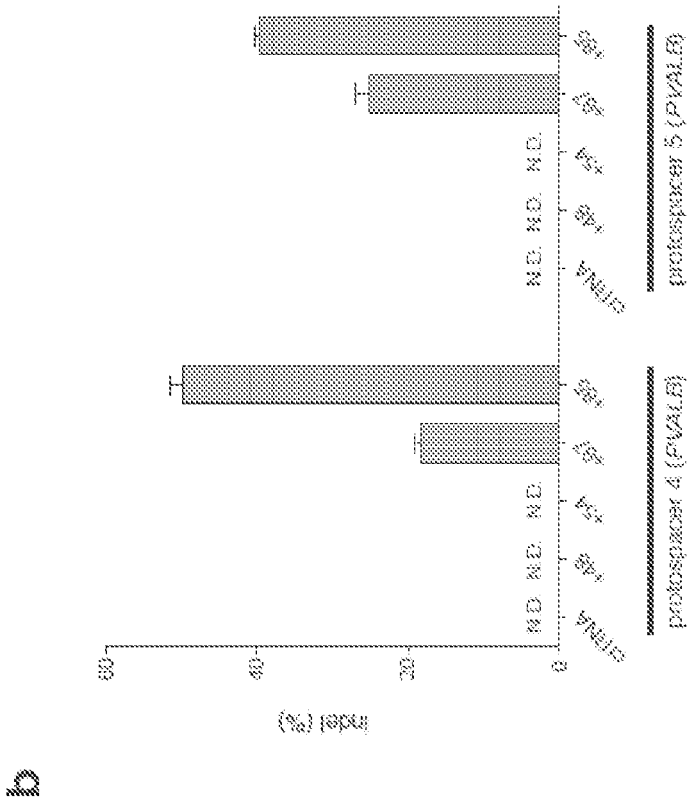

C
protospacer 4 (PVALB)
protospacer 5 (PVALB)
crRNA
+48
+54
+67
+85
crRNA
+48
+54
+67
+85
chimeric RNA
3
18.3
49.9
25.1
39.7

FIG. 16C


b
Indel (%)
0
20
40
60
protospacer 4 (PVALB)
protospacer 5 (PVALB)
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
gRNA
gRNA
gRNA
gRNA
gRNA
gRNA

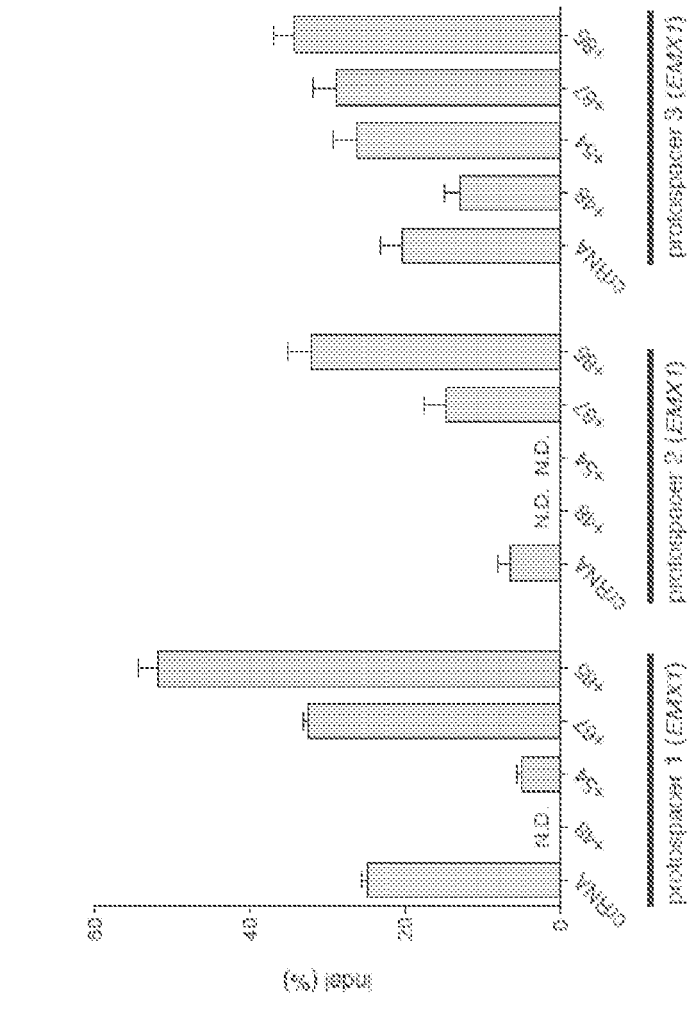

a
Indel (%)
0
20
40
60
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
N.D.
N.D.
gRNA
gRNA
gRNA
gRNA
gRNA
gRNA

FIG. 17A-B

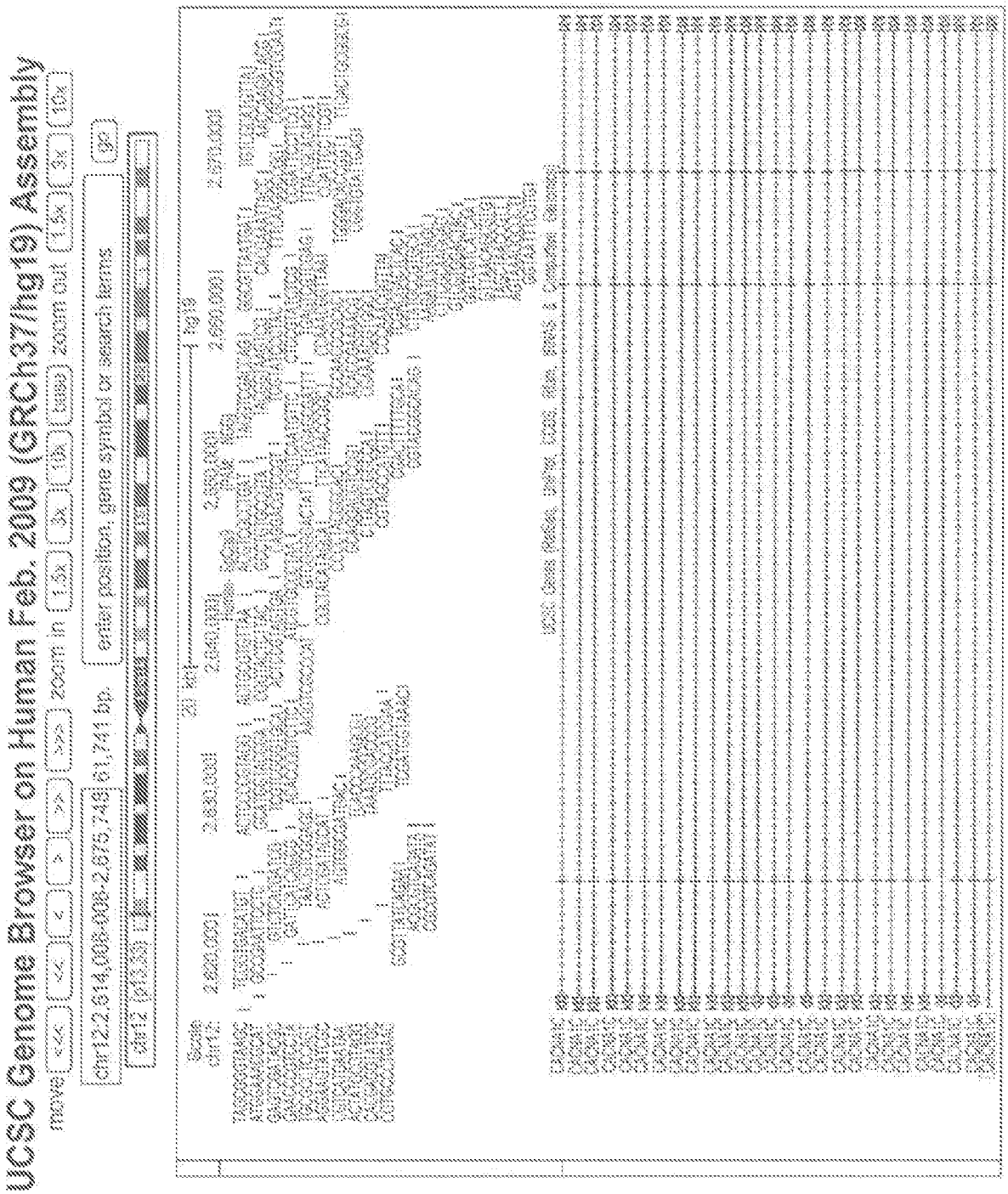

UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly
move
zoom in
zoom out
enter position, gene symbol or search terms
go
chr12:2,614,008-2,675,749 61,741 bp.
20 kb
hg19
Scale
chr12:
2,620,000
2,640,000
2,660,000
2,680,000
2,700,000
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)

FIG. 18



FIG. 19A



FIG. 19B

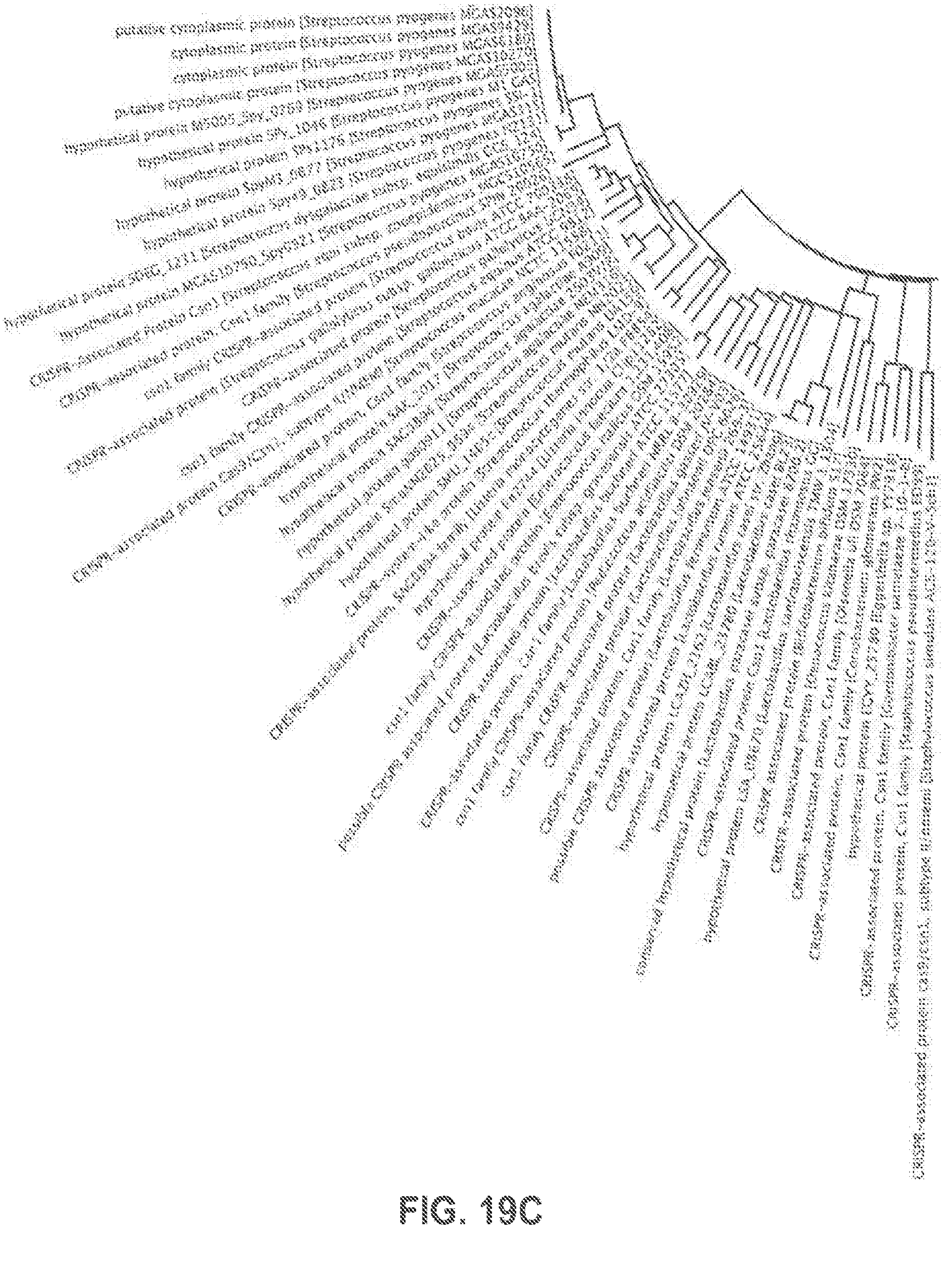


FIG. 19C



FIG. 19D

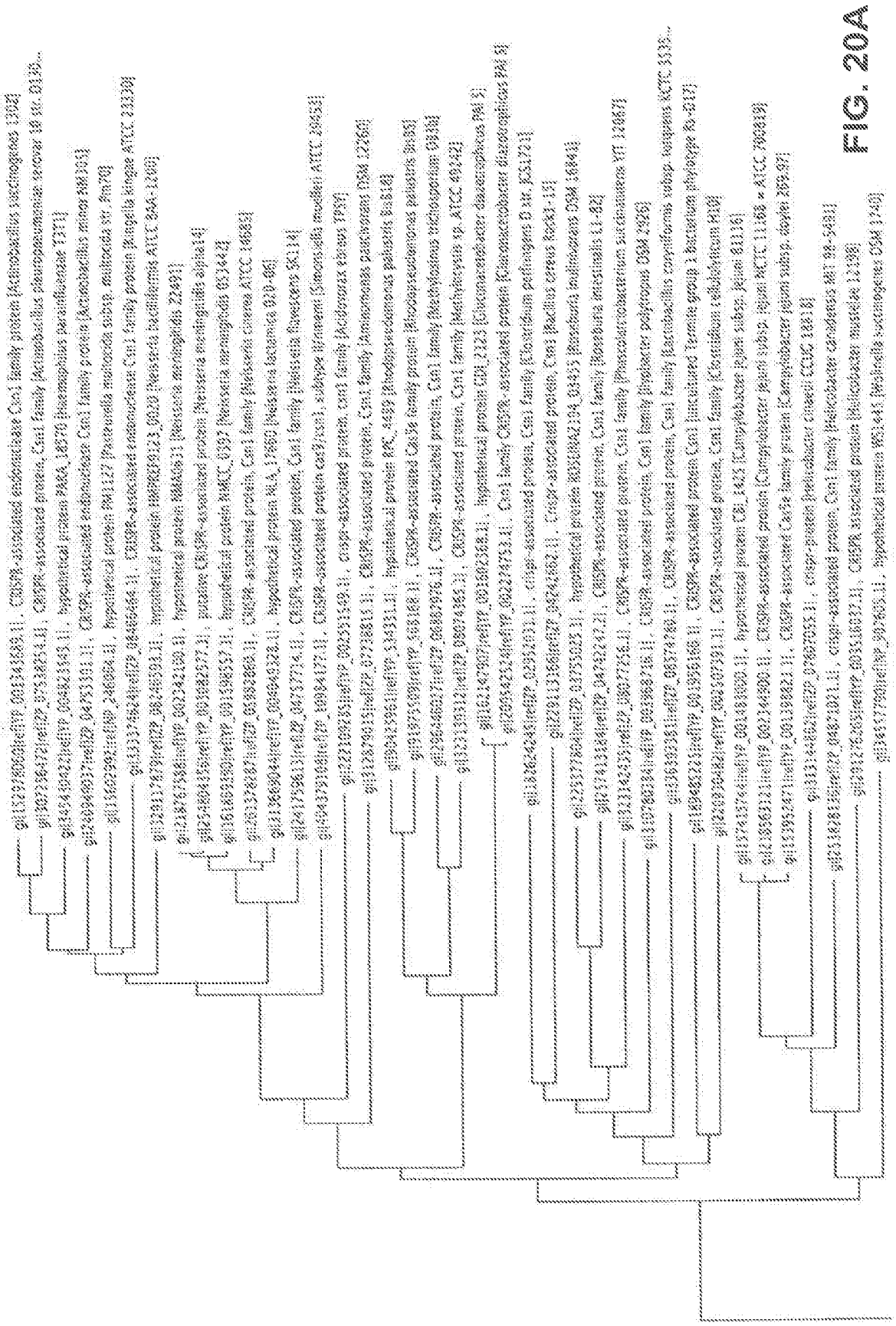


FIG. 20A



FIG. 20B

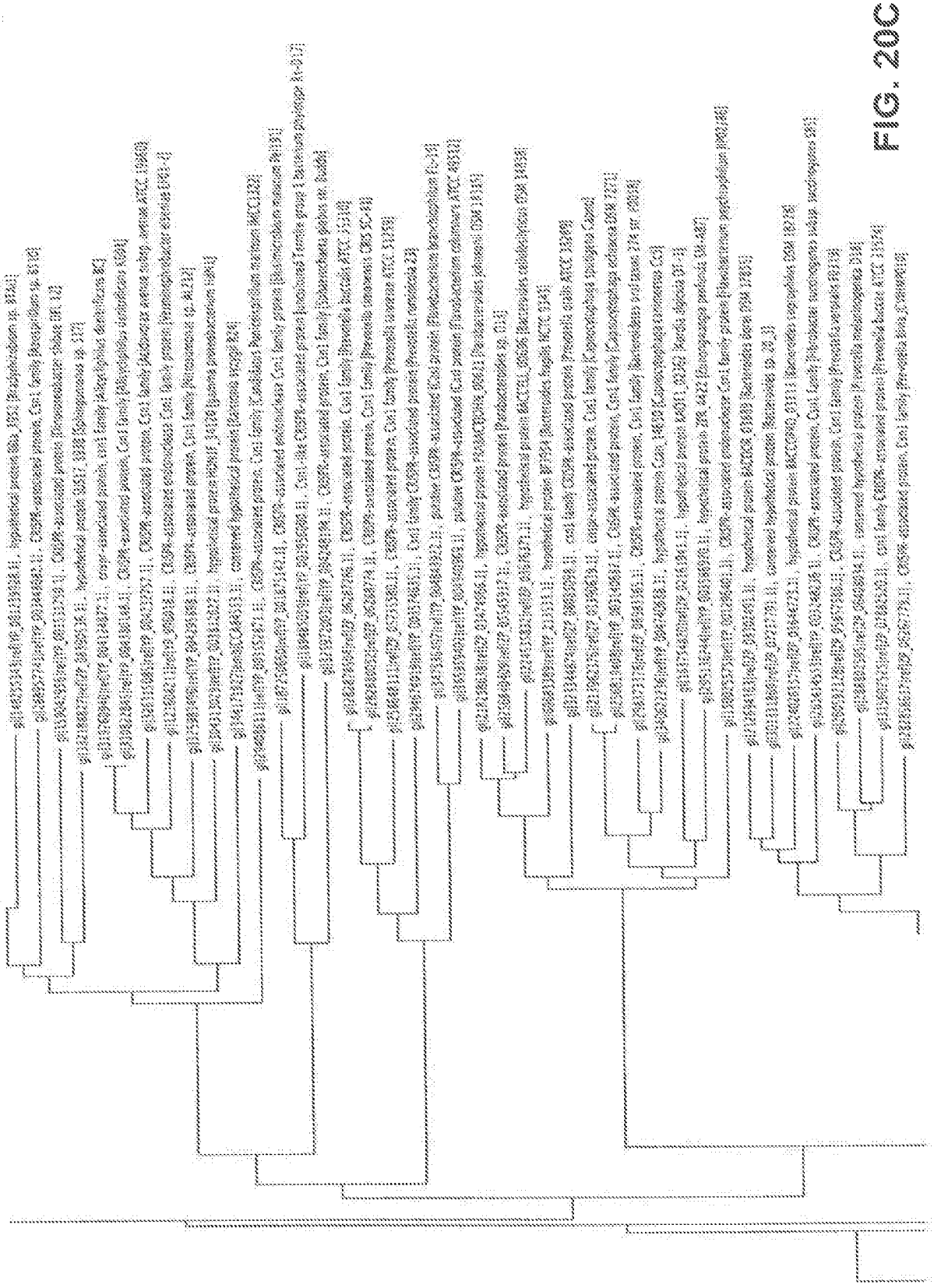


FIG. 20C

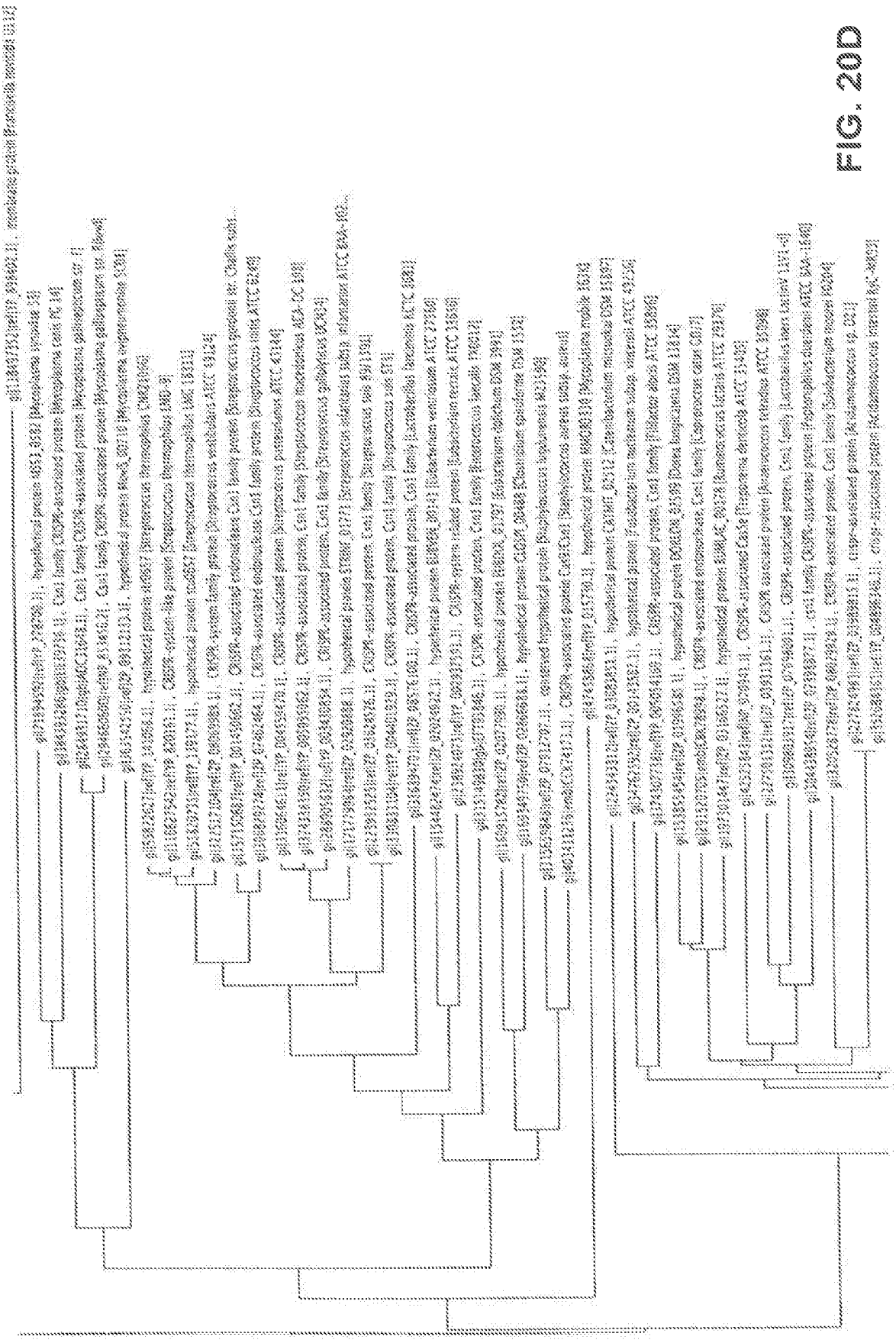


FIG. 20D

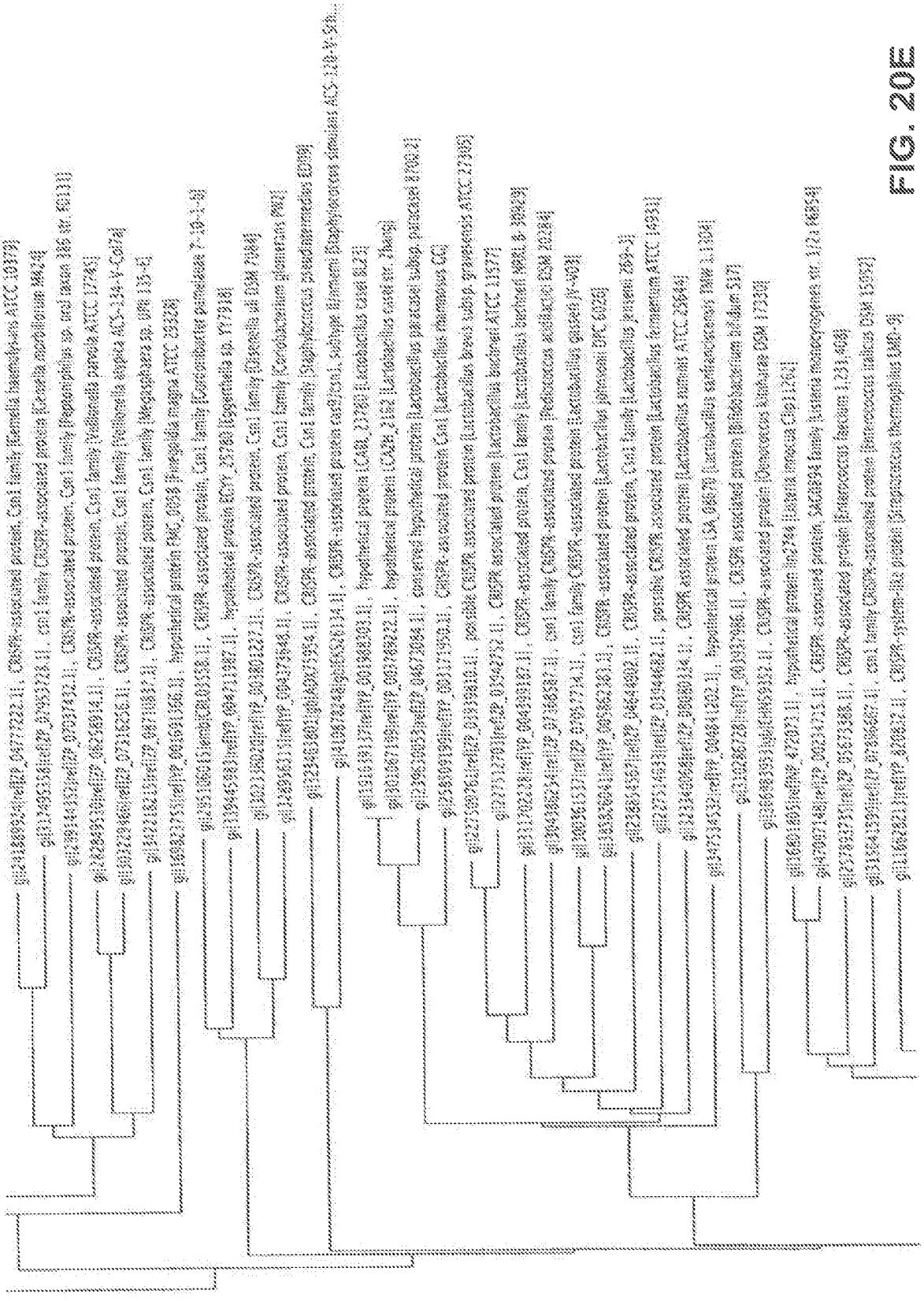


FIG. 20E

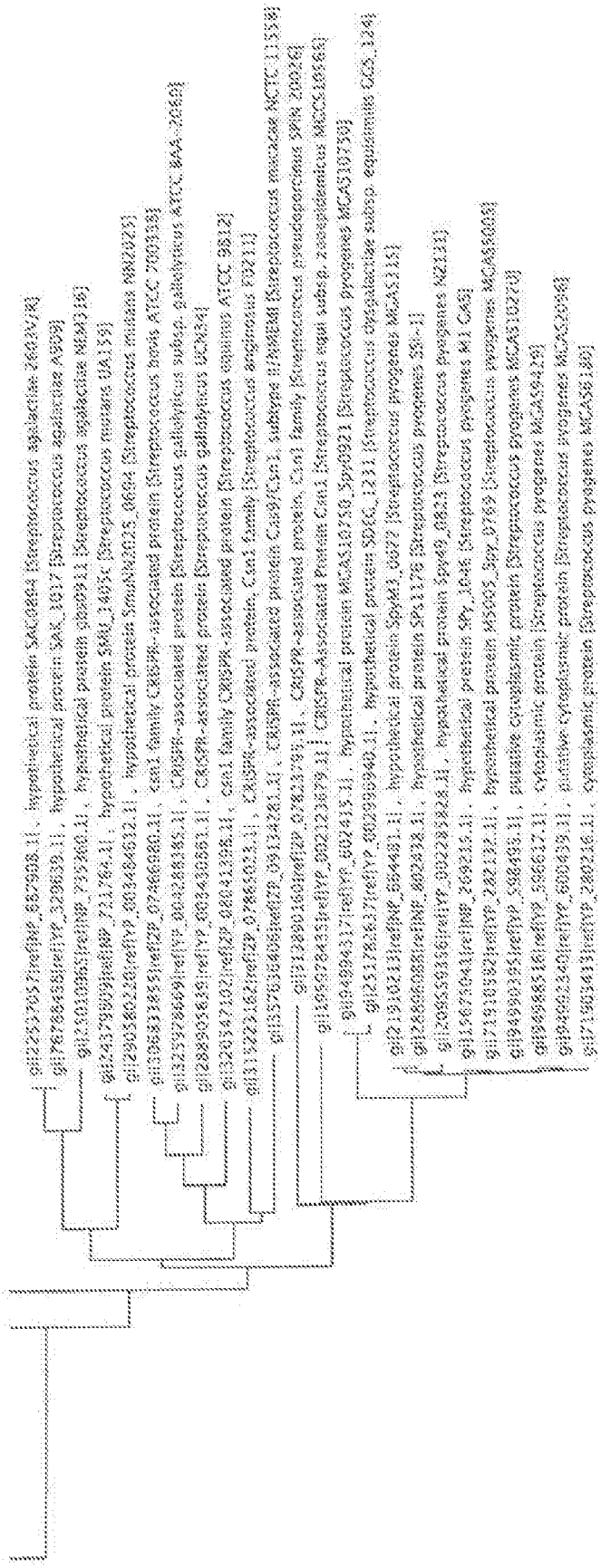


FIG. 20F

a

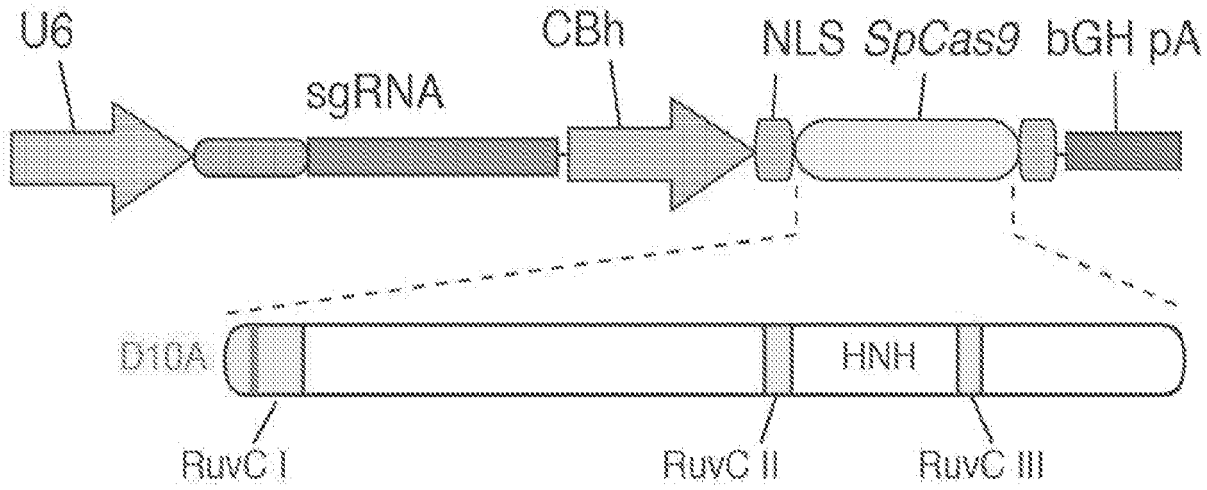


FIG. 21A

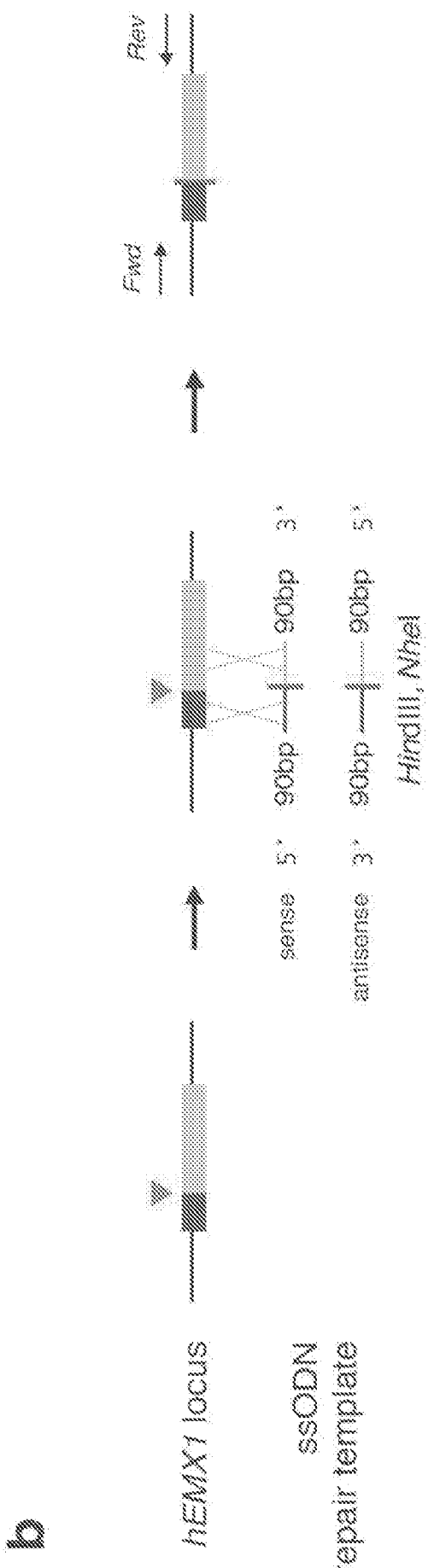

b
hEMX1 locus
ssODN
repair template
sense
antisense
5'
3'
90bp
90bp
90bp
90bp
3'
5'
HindIII, NheI
Fwd
Rev

FIG. 21B

C
hEMX1 locus
genomic target
PAM
5'- CAGAAGAAGAAGGGC
CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGTGGGCAAC
CTCTGGCCACTCCCT -3'
3'- GTCTTCTTCTTCCCG
GGTTACCCCTCCTGTAGCTACAGTGGAGGTTACTGATCCCACCACCCGTTG
GAGACCGGTGAGGGA -5'
sense
5'- CAGAAGAAGAAGGGC
ACATCGATGTCACCTCCAATGACAAGCTTGCTAGCGGTGGGCAACCACAAAC
CTCTGGCCACTCCCT -3'
HindIII
NheI
antisense
3'- GTCTTCTTCTTCCCG
TGTAGCTACAGTGGAGGTTACTGTTCGAACGATCGCCACCCGTTGGTGTTTG
GAGACCGGTGAGGGA -5'
HindIII
NheI

FIG. 21C

d

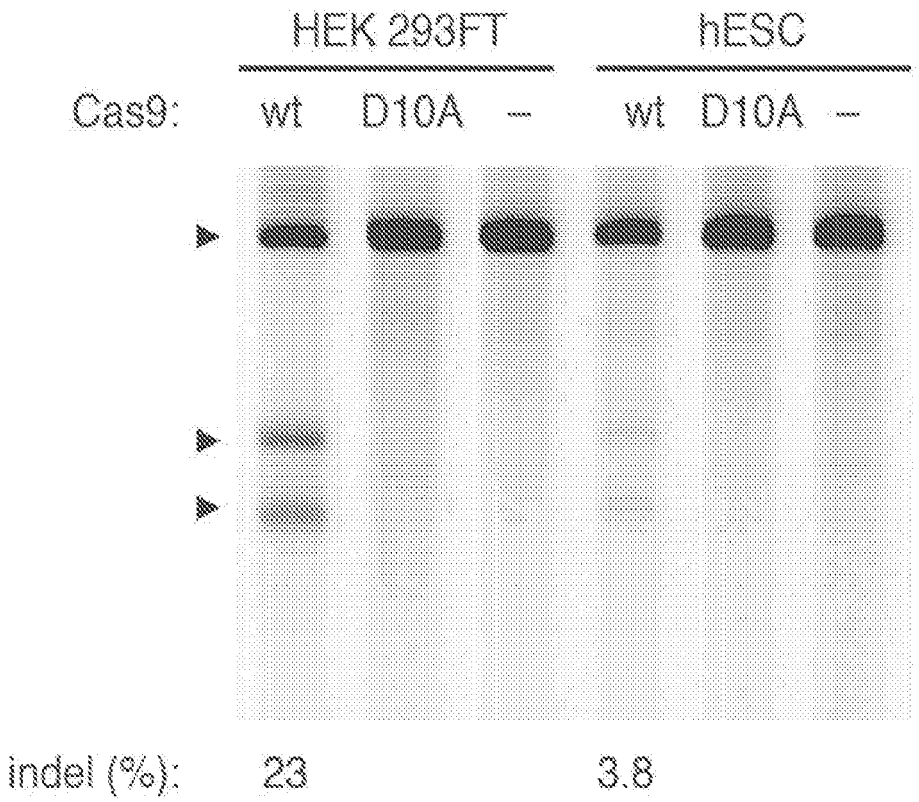


FIG. 21D

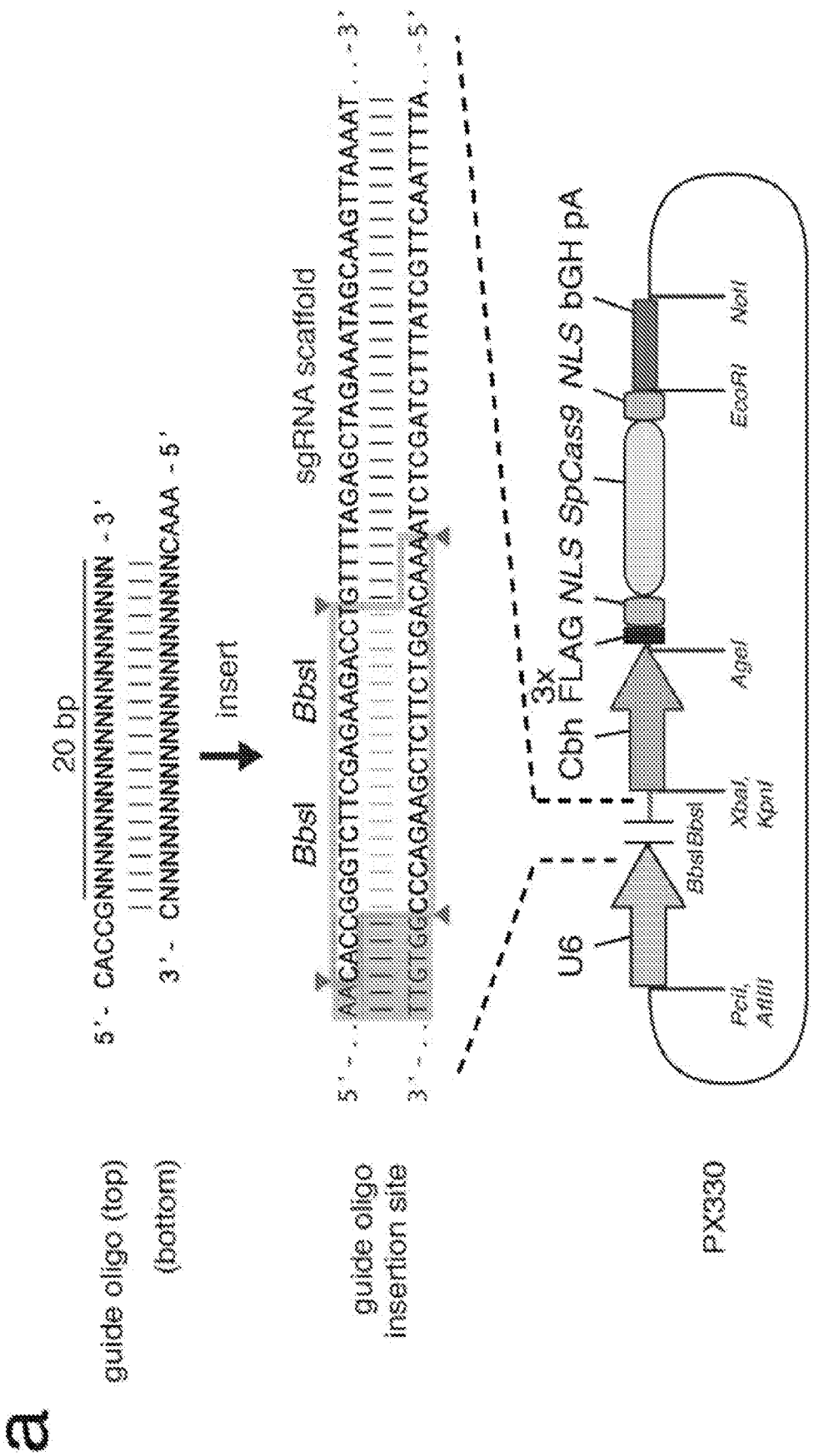

a
guide oligo (top)
(bottom)
20 bp
5' - CACCGNNNNNNNNNNNNNNNNNNN - 3'
3' - CNNNNNNNNNNNNNNNNNNNNCAAA - 5'
insert
BbsI
BbsI
sgRNA scaffold
guide oligo insertion site
5' - . . . AACACCGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT . . . 3'
3' - . . . TTGTGGCCCAGAAGCTCTTCTGGACAAAATCTCGATCTTTATCGTTCAATTTTA . . . 5'
3x
Cbh FLAG NLS SpCas9 NLS bGH pA
U6
PX330
PenI, AflIII
BbsI BbsI
XbaI, KpnI
AgeI
EcoRI
NotI

FIG. 22A

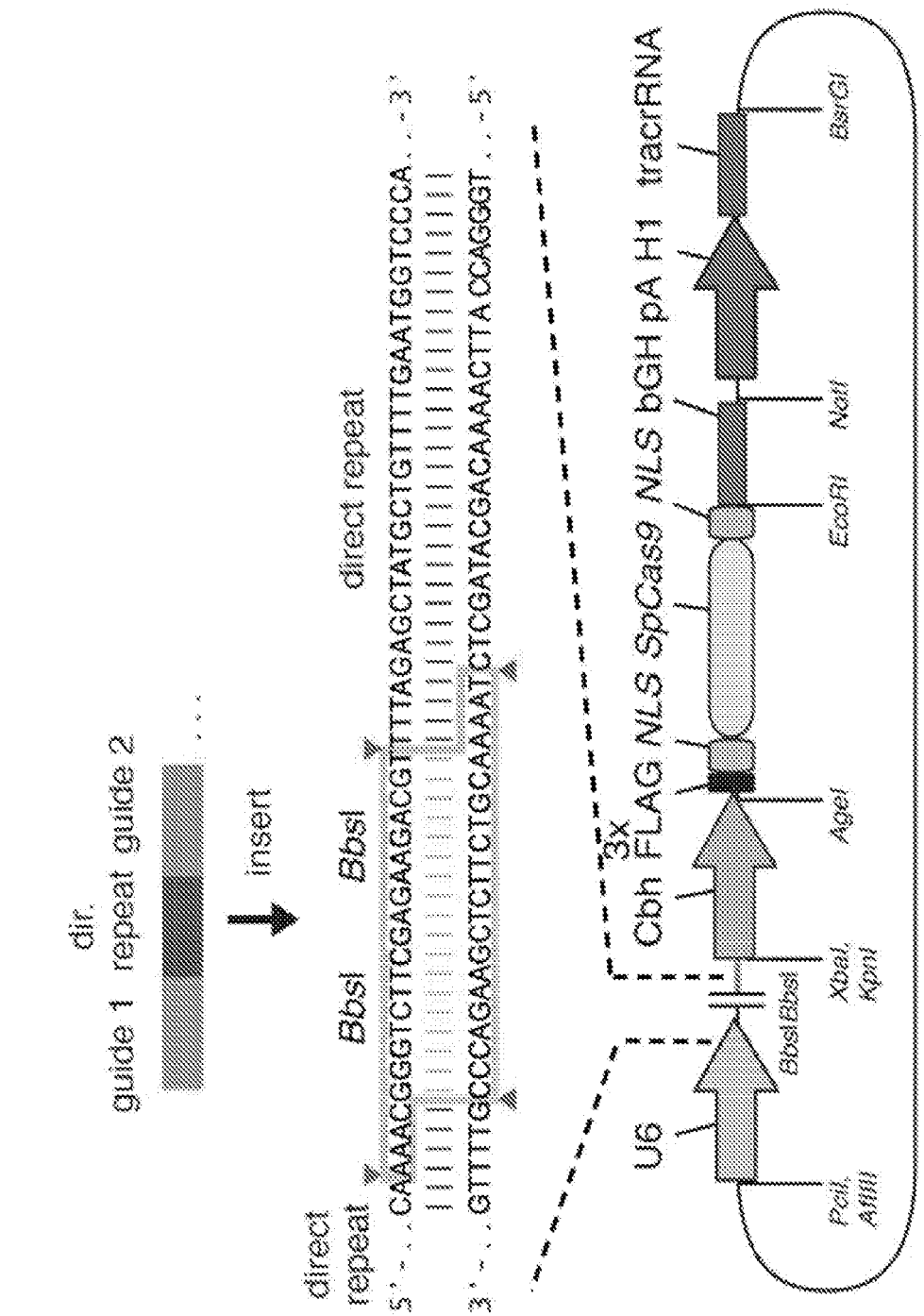

b
dir. repeat guide 1 guide 2
insert
BbsI
BbsI
direct repeat
direct repeat
5' - . . . CAAAACGGGTCTTCGAGAAGACGTTTTAGAGCTATGCTGTTTTGAATGGTCCCA. . . - 3'
3' - . . . GTTTTGCCCAGAAGCTCTTCTGCAAAATCTCGATACGACAAAACTTACCAGGGT . . . - 5'
guide oligo insertion site
U6
3x
Cbh FLAG NLS SpCas9 NLS bGH pA H1 tracrRNA
PX260
PciI, AflIII
BbsI BbsI
XbaI, KpnI
AgeI
EcoRI
NotI
BsrGI

FIG. 22B

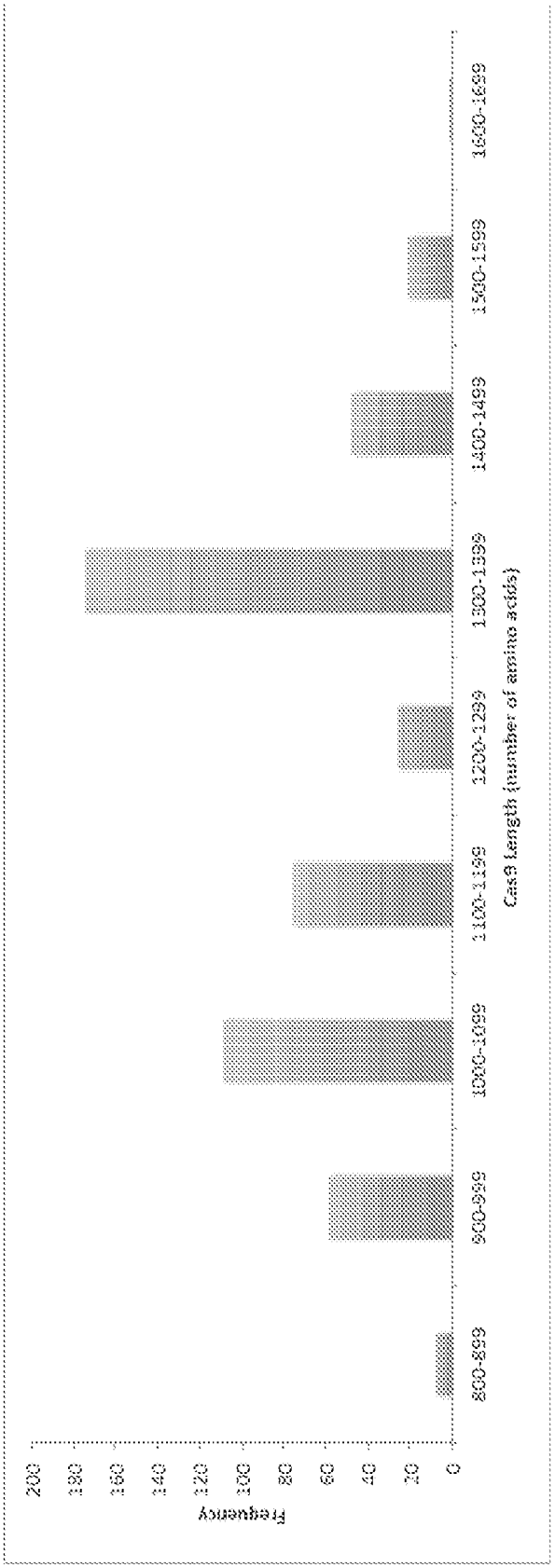

Frequency
0
20
40
60
80
100
120
140
160
180
200
800-899
900-999
1000-1099
1100-1199
1200-1299
1300-1399
1400-1499
1500-1599
1600-1699
Cas9 Length (number of amino acids)

FIG. 23

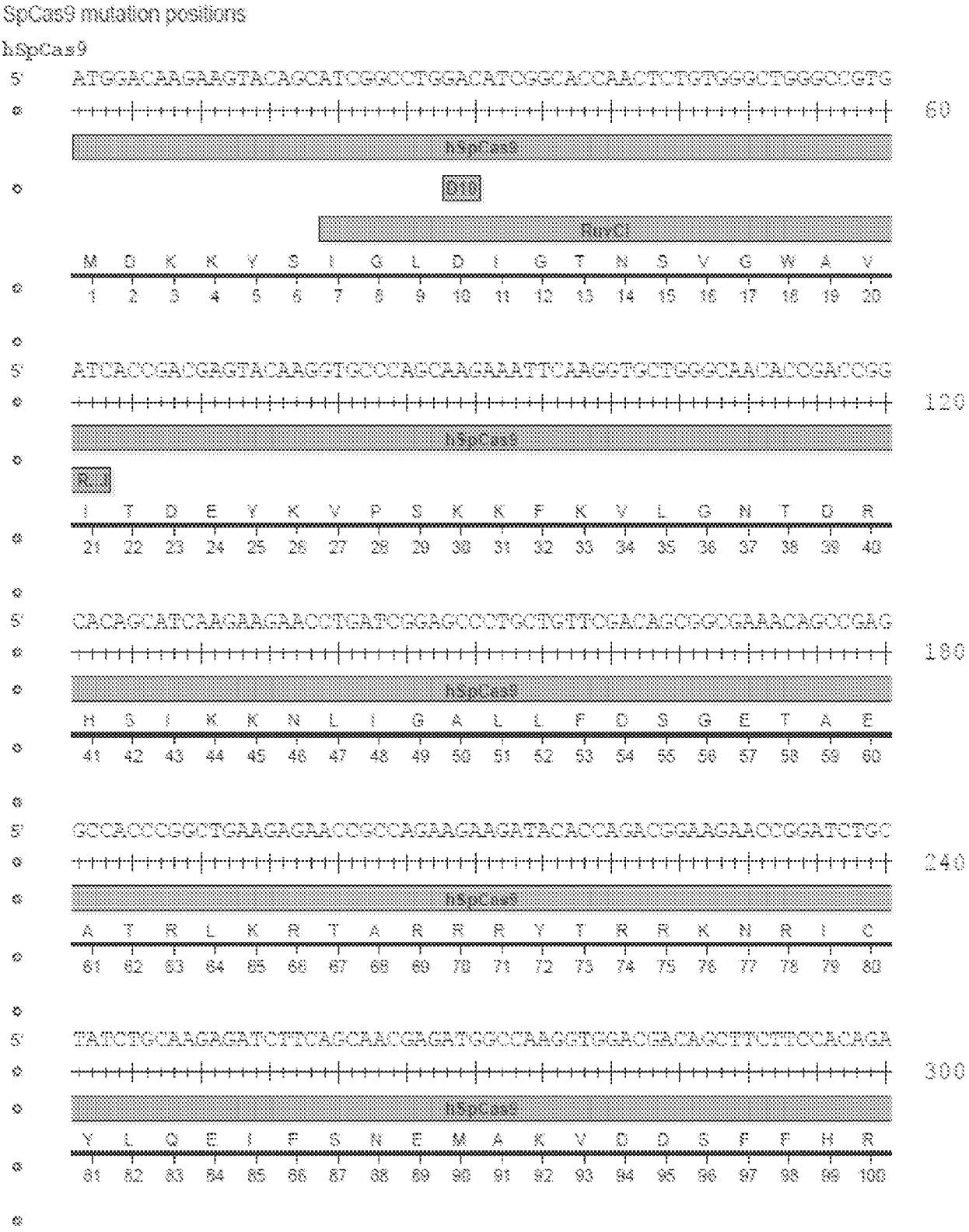

SpCas9 mutation positions
hSpCas9
5'
ATGGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTG
60
hSpCas9
RuvC1
M D K K Y S I G L D I G T N S V G W A V
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5'
ATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGG
120
hSpCas9
I T D E Y K V P S K K F K V L G N T D R
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
5'
CACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAG
180
hSpCas9
H S I K K N L I G A L L F D S G E T A E
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
5'
GCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC
240
hSpCas9
A T R L K R T A R R R Y T R R K N R I C
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
5'
TATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGA
300
hSpCas9
Y L Q E I F S N E M A K V D D S F F H R
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

FIG. 24A

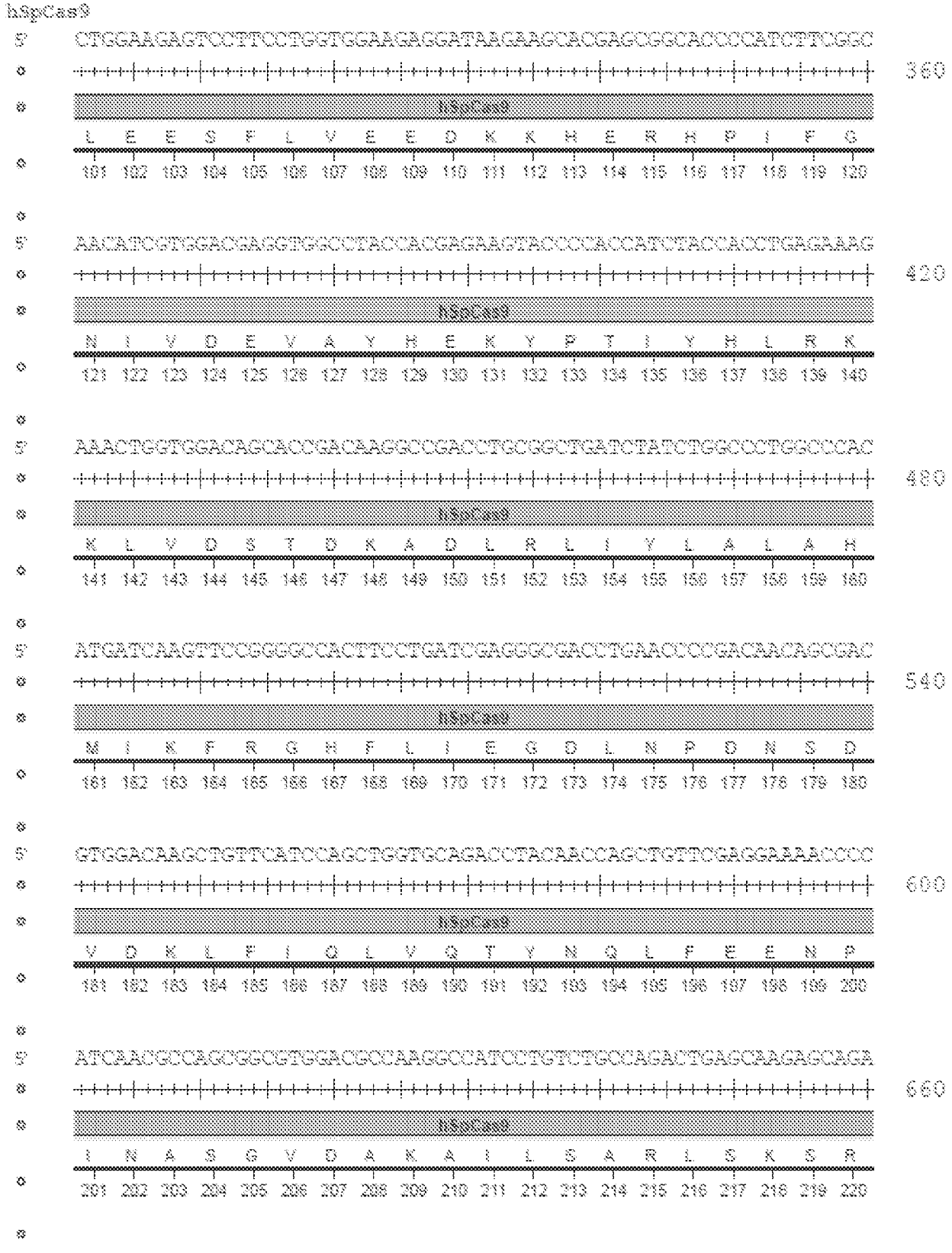

hSpCas9
5' CTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGC
360
hSpCas9
L E E S F L V E E D K K H E R H P I F G
101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
5' AACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAG
420
hSpCas9
N I V D E V A Y H E K Y P T I Y H L R K
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
5' AAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCAC
480
hSpCas9
K L V D S T D K A D L R L I Y L A L A H
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
5' ATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGAC
540
hSpCas9
M I K F R G H F L I E G D L N P D N S D
161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
5' GTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCC
600
hSpCas9
V D K L F I Q L V Q T Y N Q L F E E N P
181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200
5' ATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGA
660
hSpCas9
I N A S G V D A K A I L S A R L S K S R
201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220

FIG. 24B

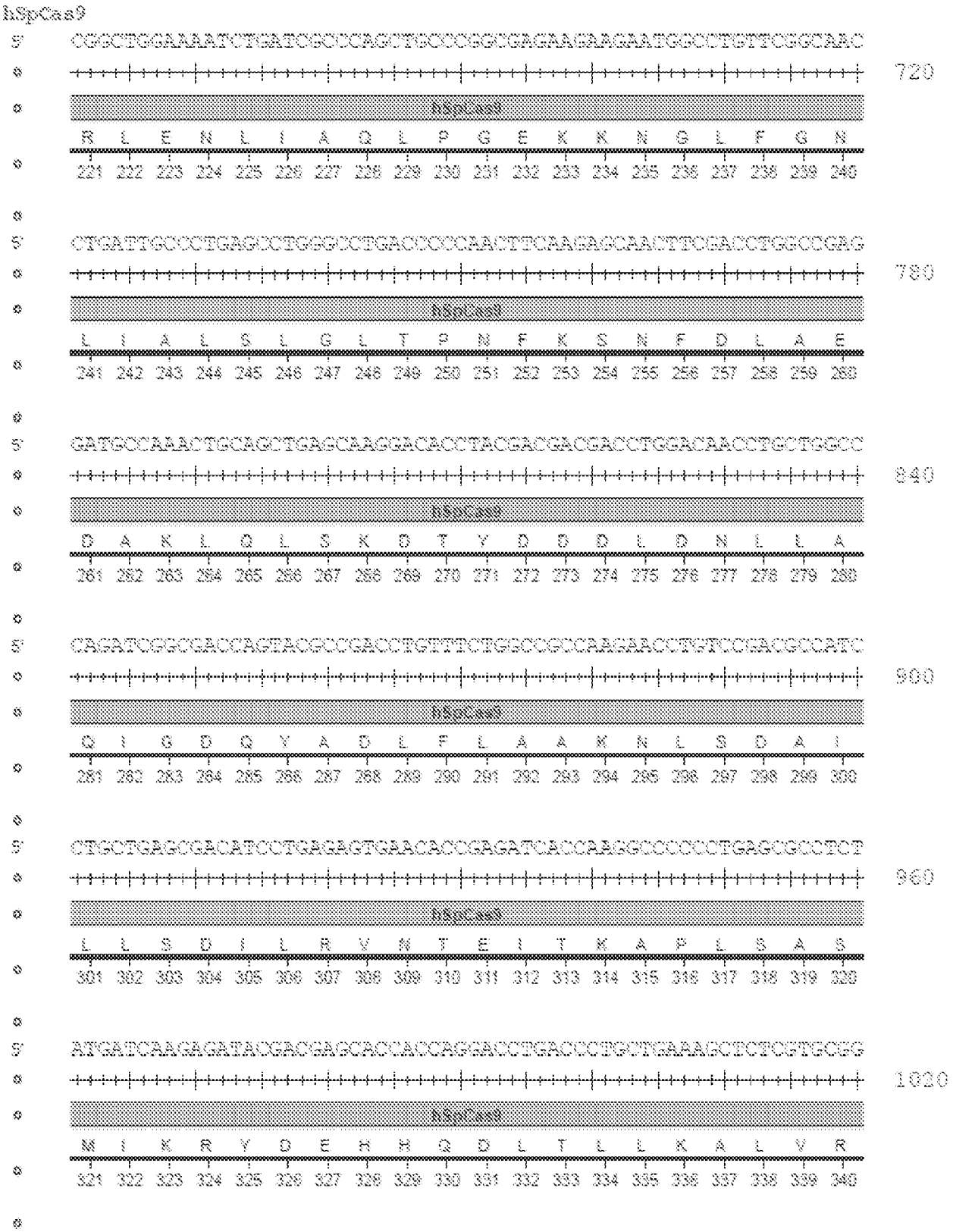

hSpCas9
5' CGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGCAAC
720
hSpCas9
R L E N L I A Q L P G E K K N G L F G N
221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240
5' CTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAG
780
hSpCas9
L I A L S L G L T P N F K S N F D L A E
241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260
5' GATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCC
840
hSpCas9
D A K L Q L S K D T Y D D D L D N L L A
261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280
5' CAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATC
900
hSpCas9
Q I G D Q Y A D L F L A A K N L S D A I
281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300
5' CTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCT
960
hSpCas9
L L S D I L R V N T E I T K A P L S A S
301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320
5' ATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGG
1020
hSpCas9
M I K R Y D E H H Q D L T L L K A L V R
321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340

FIG. 24C

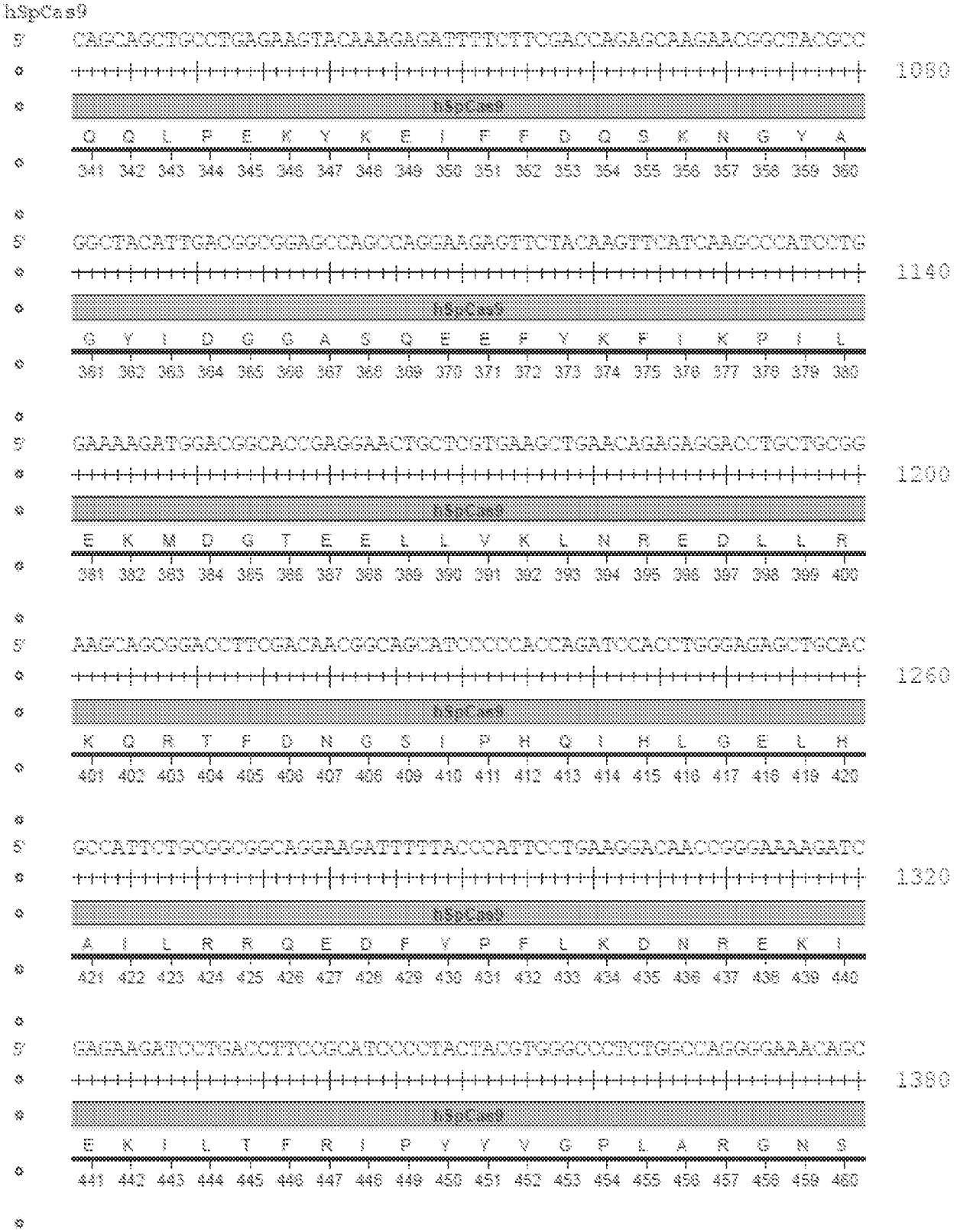

hSpCas9
5' CAGCAGCTGCCTGAGGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCC
1080
hSpCas9
Q Q L P E K Y K E I F F D Q S K N G Y A
341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360
5' GGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTG
1140
hSpCas9
G Y I D G G A S Q E E F Y K F I K P I L
361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380
5' GAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
1200
hSpCas9
E K M D G T E E L L V K L N R E D L L R
381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400
5' AAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCAC
1260
hSpCas9
K Q R T F D N G S I P H Q I H L G E L H
401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420
5' GCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATC
1320
hSpCas9
A I L R R Q E D F Y P F L K D N R E K I
421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440
5' GAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGC
1380
hSpCas9
E K I L T F R I P Y Y V G P L A R G N S
441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460

FIG. 24D

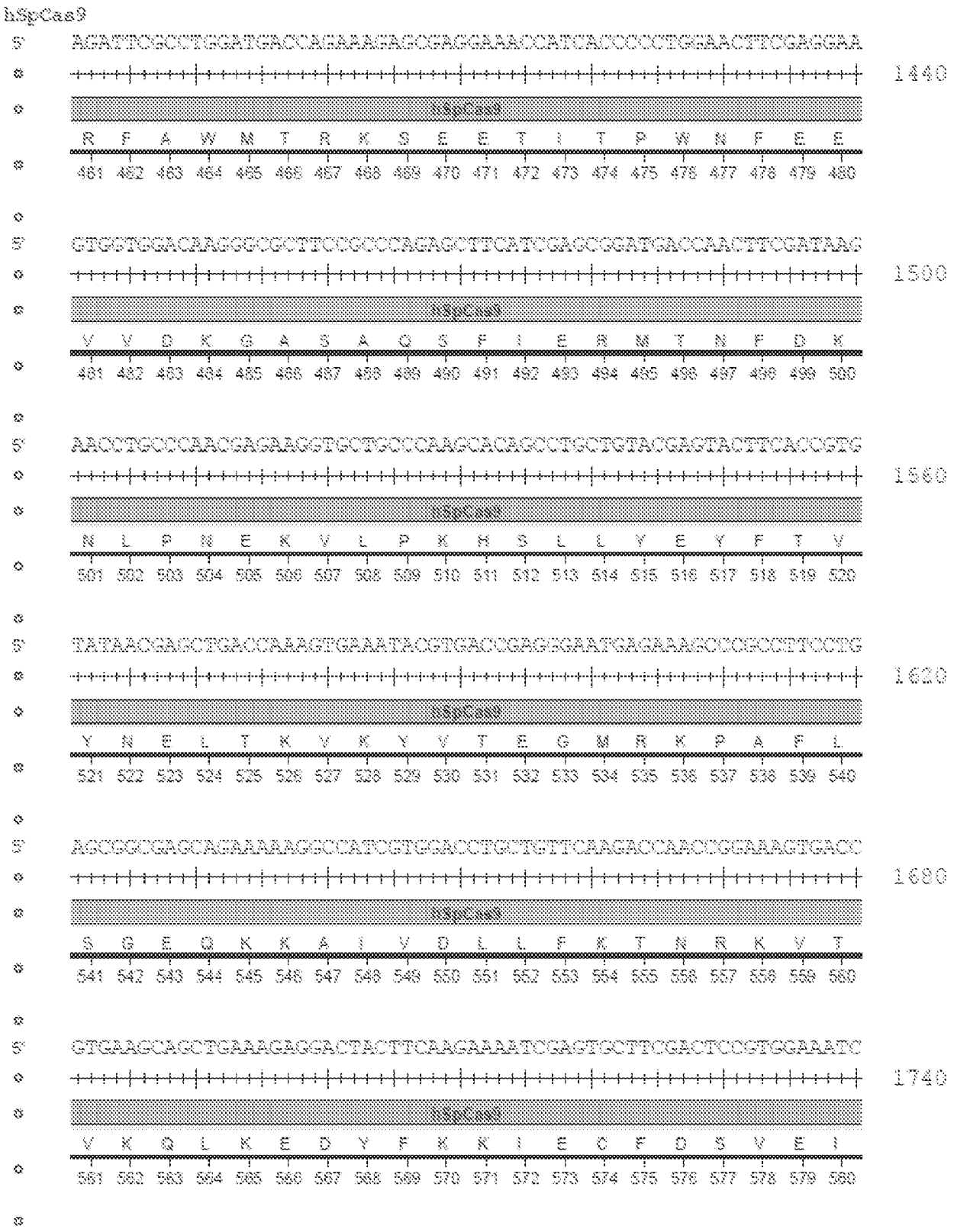

hSpCas9
5' AGATTCGCCTGGATGACCATAAAGATGCGAGGAAACCATCACCCCTGGAACTTCGAGGAA
1440
hSpCas9
R F A W M T R K S E E T I T P W N F E E
481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500
5' GTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAG
1500
hSpCas9
V V D K G A S A Q S F I E R M T N F D K
481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500
5' AACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTG
1560
hSpCas9
N L P N E K V L P K H S L L Y E Y F T V
501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520
5' TATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTCCTG
1620
hSpCas9
Y N E L T K V K Y V T E G M R K P A F L
521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540
5' AGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACC
1680
hSpCas9
S G E Q K K A I V D L L F K T N R K V T
541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560
5' GTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATC
1740
hSpCas9
V K Q L K E D Y F K K I E C F D S V E I
561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580

FIG. 24E

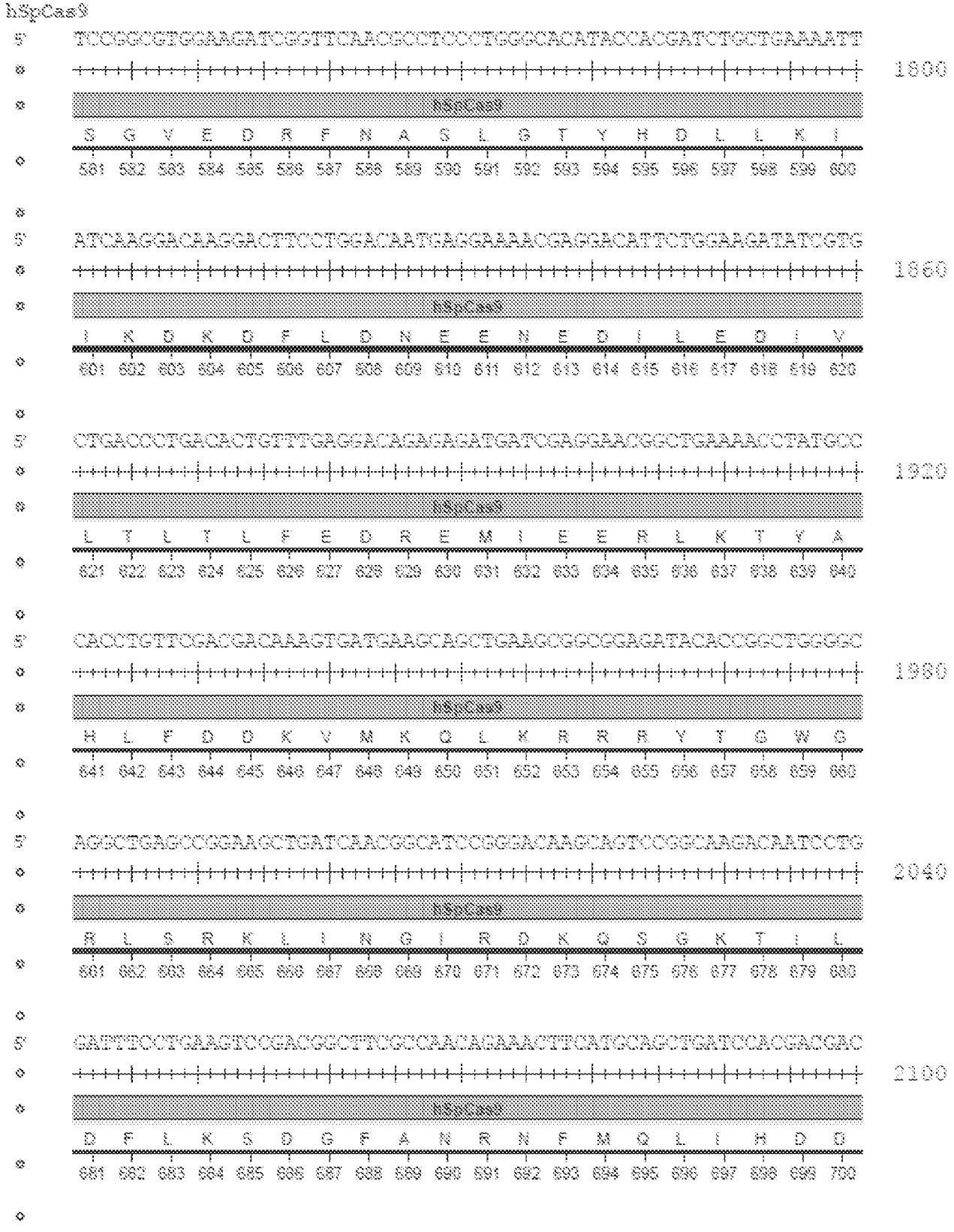

hSpCas9
5' TCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATT
1800
hSpCas9
S G V E D R F N A S L G T Y H D L L K I
581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600
5' ATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTG
1860
hSpCas9
I K D K D F L D N E E N E D I L E D I V
601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620
5' CTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCC
1920
hSpCas9
L T L T L F E D R E M I E E R L K T Y A
621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640
5' CACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGC
1980
hSpCas9
H L F D D K V M K Q L K R R R Y T G W G
641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660
5' AGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTG
2040
hSpCas9
R L S R K L I N G I R D K Q S G K T I L
661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680
5' GATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGACGAC
2100
hSpCas9
D F L K S D G F A N R N F M Q L I H D D
681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700

FIG. 24F

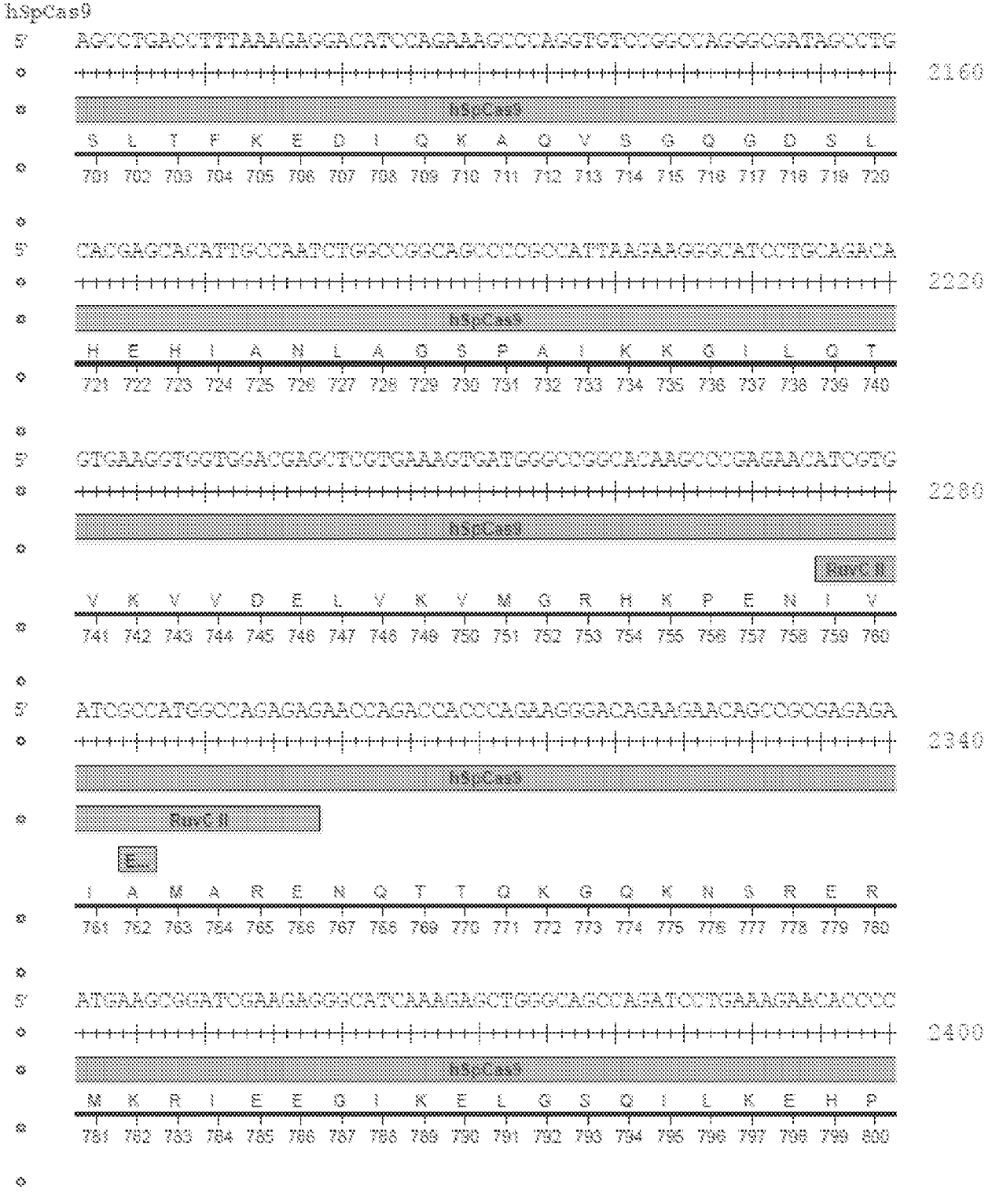

hSpCas9
5' AGCCTGACCTTTAAAGAGGACATCCAGAAAGCCCAGGTGTCCGGCCAGGGCGATAGCCTG
2160
hSpCas9
S L T F K E D I Q K A Q V S G Q G D S L
701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720
5' CACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACA
2220
hSpCas9
H E H I A N L A G S P A I K K G I L Q T
721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740
5' GTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTG
2280
hSpCas9
SpyC N
V K V V D E L V K V M G R H K P E N I V
741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760
5' ATCGCCATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGA
2340
hSpCas9
SpyC N
E...
I A M A R E N Q T T Q K G Q K N S R E R
761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780
5' ATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCC
2400
hSpCas9
M K R I E E G I K E L G S Q I L K E H P
781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800

FIG. 24G

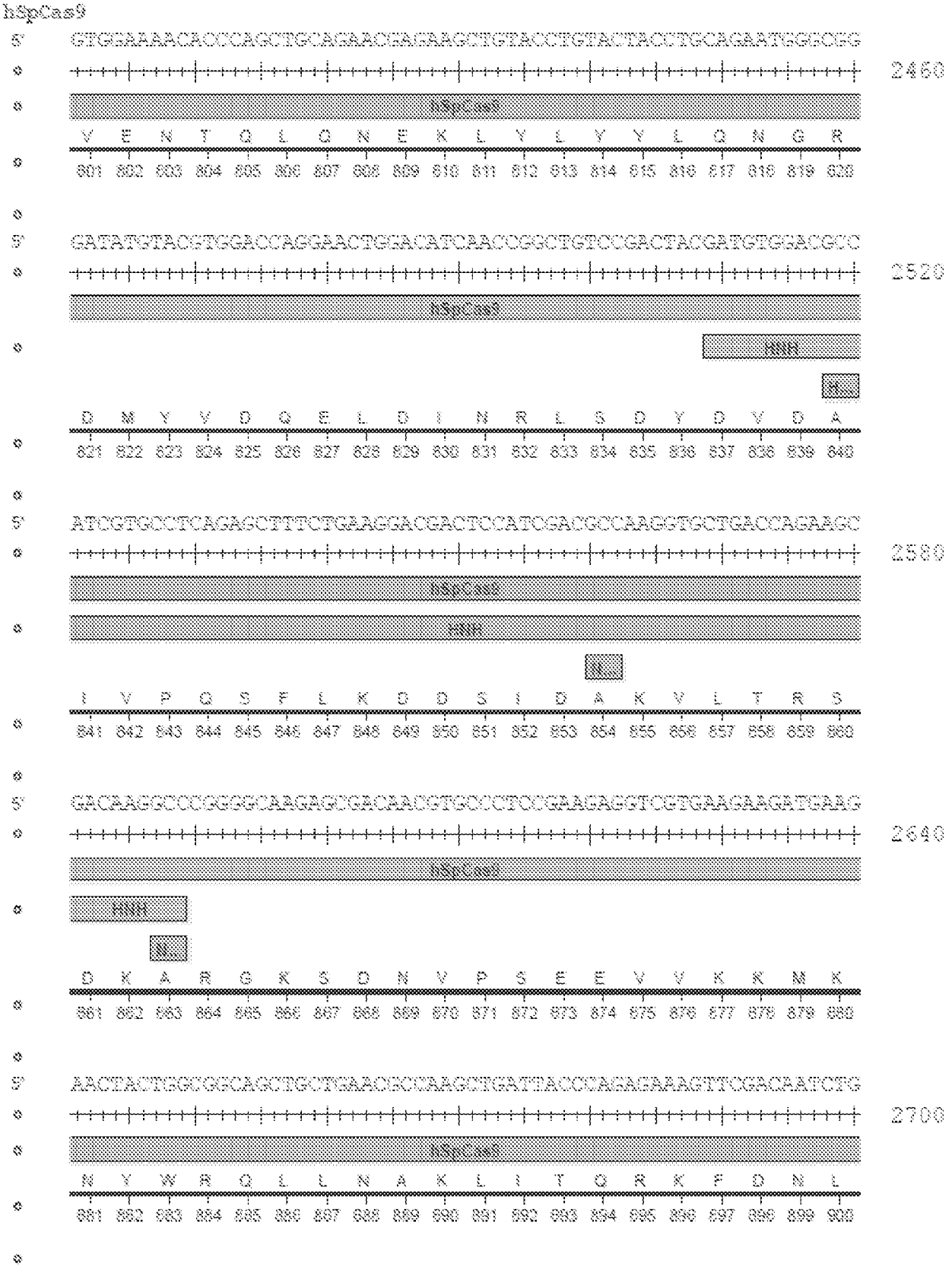

hSpCas9
5'
GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGG
2460
hSpCas9
V E N T Q L Q N E K L Y L Y Y L Q N G R
801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820
5'
GATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACGCC
2520
hSpCas9
HNH
NLS
D M Y V D Q E L D I N R L S D Y D V D A
821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840
5'
ATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACGCCAAGGTGCTGACCAGAAGC
2580
hSpCas9
HNH
NLS
I V P Q S F L K D D S I D A K V L T R S
841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860
5'
GACAAGGCCCTGGGGCAAGAGGTGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAG
2640
hSpCas9
HNH
NLS
D K A R G K S D N V P S E E V V K K M K
861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880
5'
AACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTG
2700
hSpCas9
N Y W R Q L L N A K L I T Q R K F D N L
881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900

FIG. 24H

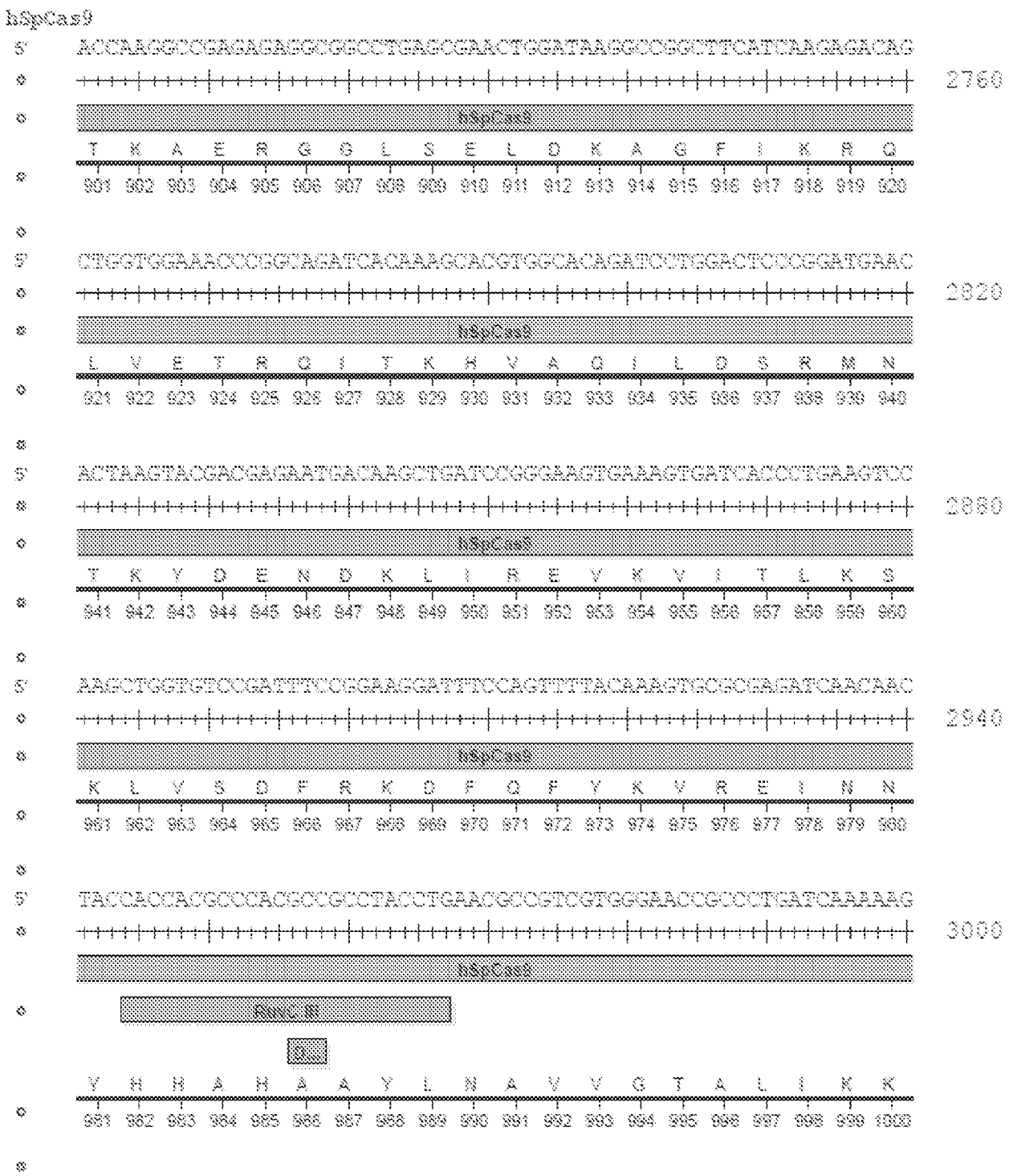

hSpCas9
5'
ACCAAGGCCGAGAGAAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG
2760
hSpCas9
T K A E R G G L S E L D K A G F I K R Q
901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920
5'
CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAAC
2820
hSpCas9
L V E T R Q I T K H V A Q I L D S R M N
921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940
5'
ACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGTCC
2880
hSpCas9
T K Y D E N D K L I R E V K V I T L K S
941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960
5'
AAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAAC
2940
hSpCas9
K L V S D F R K D F Q F Y K V R E I N N
961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980
5'
TACCACCACGCCCACGACGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAG
3000
hSpCas9
RuvC III
H
Y H H A H A Y L N A V V G T A L I K K
981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000

FIG. 24I

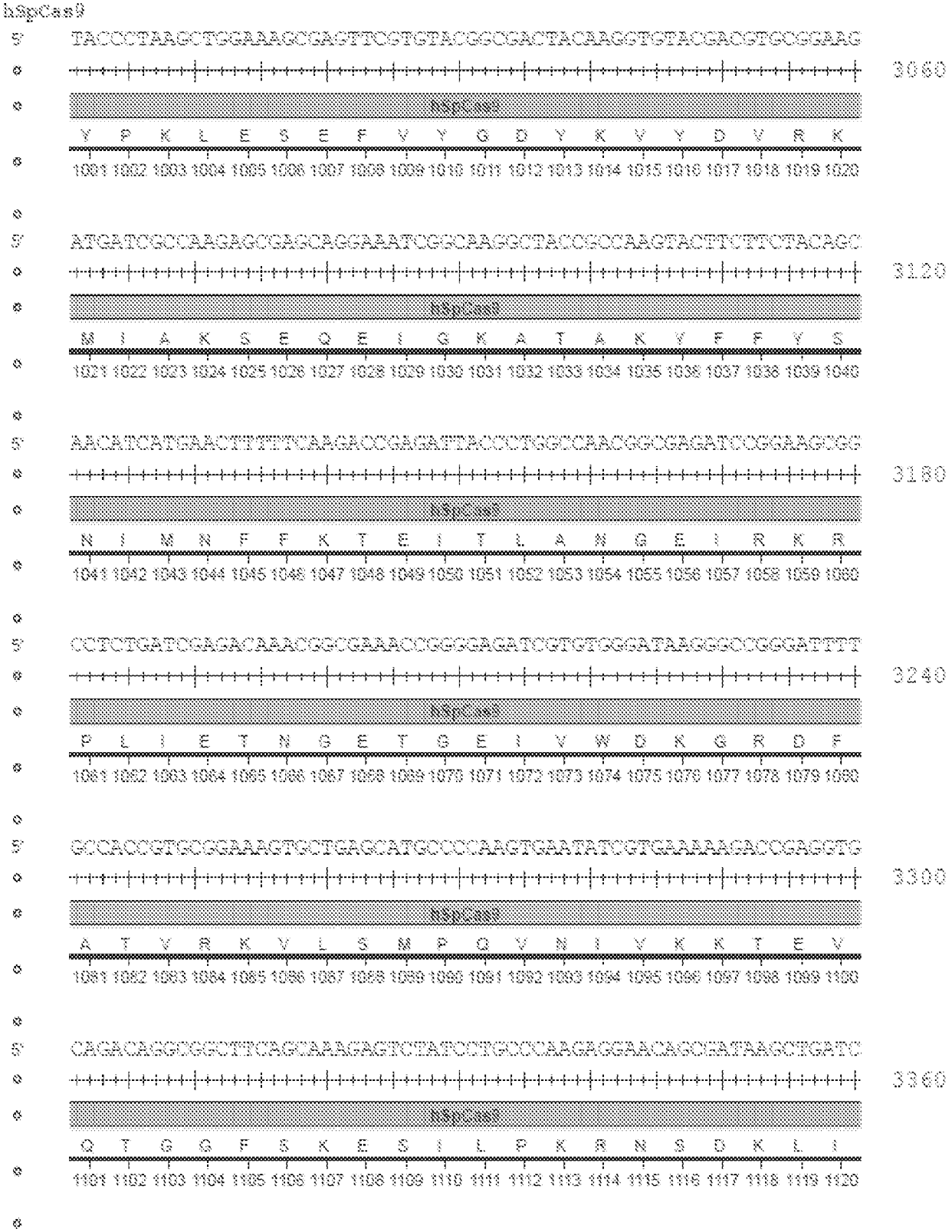

hSpCas9
5' TACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAG
3060
hSpCas9
Y P K L E S E F V Y G D Y K V Y D V R K
1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020
5' ATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGC
3120
hSpCas9
M I A K S E Q E I G K A T A K Y F F Y S
1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040
5' AACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGG
3180
hSpCas9
N I M N F F K T E I T L A N G E I R K R
1041 1042 1043 1044 1045 1046 1047 1048 1049 1050 1051 1052 1053 1054 1055 1056 1057 1058 1059 1060
5' CCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTT
3240
hSpCas9
P L I E T N G E T G E I V W D K G R D F
1061 1062 1063 1064 1065 1066 1067 1068 1069 1070 1071 1072 1073 1074 1075 1076 1077 1078 1079 1080
5' GCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTG
3300
hSpCas9
A T V R K V L S M P Q V N I V K K T E V
1081 1082 1083 1084 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094 1095 1096 1097 1098 1099 1100
5' CAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATC
3360
hSpCas9
Q T G G F S K E S I L P K R N S D K L I
1101 1102 1103 1104 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118 1119 1120

FIG. 24J

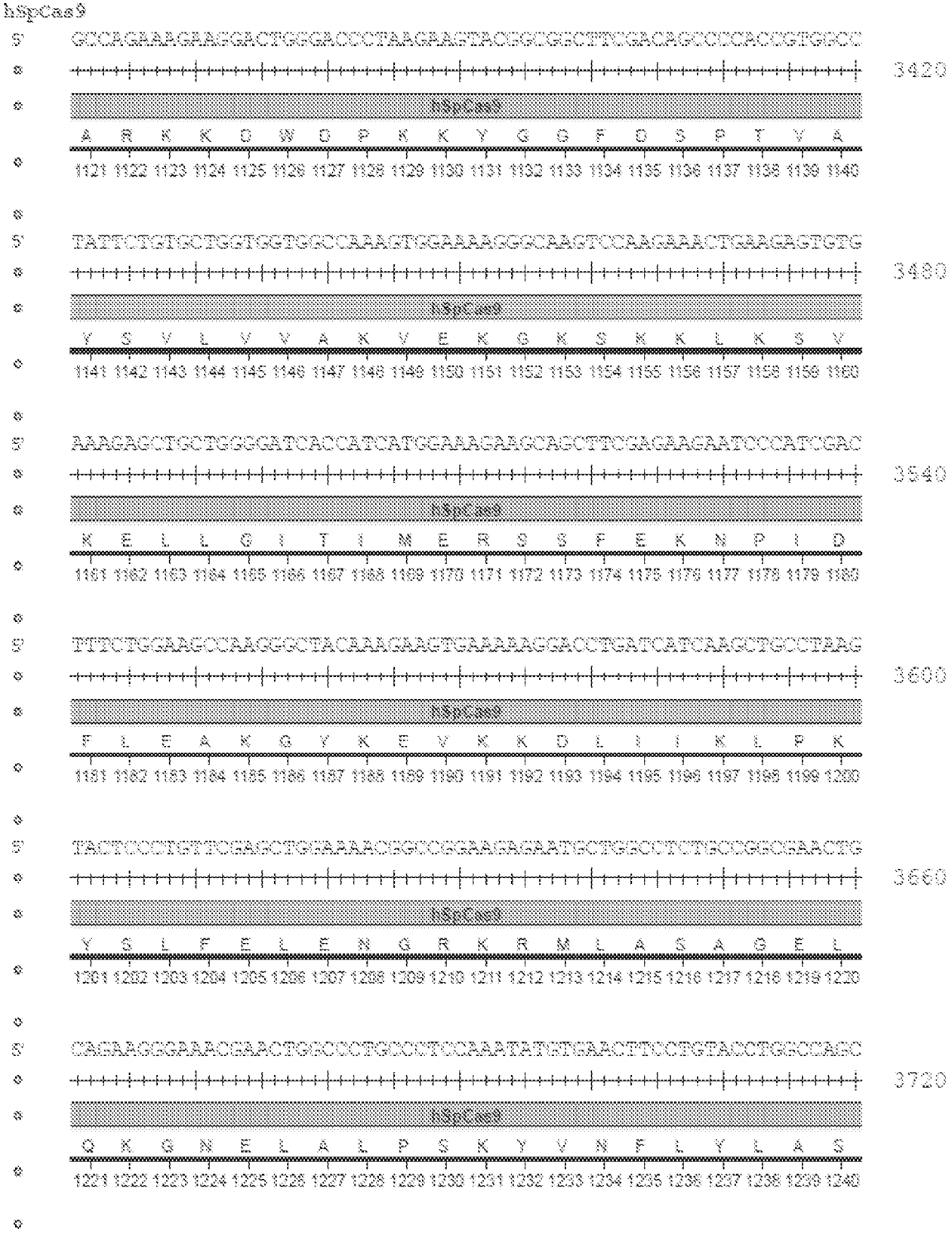

hSpCas9
5' GCCAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCC
3420
hSpCas9
A R K K D W D P K K Y G G F D S P T V A
1121 1122 1123 1124 1125 1126 1127 1128 1129 1130 1131 1132 1133 1134 1135 1136 1137 1138 1139 1140
5' TATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTG
3480
hSpCas9
Y S V L V V A K V E K G K S K K L K S V
1141 1142 1143 1144 1145 1146 1147 1148 1149 1150 1151 1152 1153 1154 1155 1156 1157 1158 1159 1160
5' AAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGAC
3540
hSpCas9
K E L L G I T I M E R S S F E K N P I D
1161 1162 1163 1164 1165 1166 1167 1168 1169 1170 1171 1172 1173 1174 1175 1176 1177 1178 1179 1180
5' TTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAG
3600
hSpCas9
F L E A K G Y K E V K K D L I I K L P K
1181 1182 1183 1184 1185 1186 1187 1188 1189 1190 1191 1192 1193 1194 1195 1196 1197 1198 1199 1200
5' TACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTG
3660
hSpCas9
Y S L F E L E N G R K R M L A S A G E L
1201 1202 1203 1204 1205 1206 1207 1208 1209 1210 1211 1212 1213 1214 1215 1216 1217 1218 1219 1220
5' CAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCAGC
3720
hSpCas9
Q K G N E L A L P S K Y V N F L Y L A S
1221 1222 1223 1224 1225 1226 1227 1228 1229 1230 1231 1232 1233 1234 1235 1236 1237 1238 1239 1240

FIG. 24K

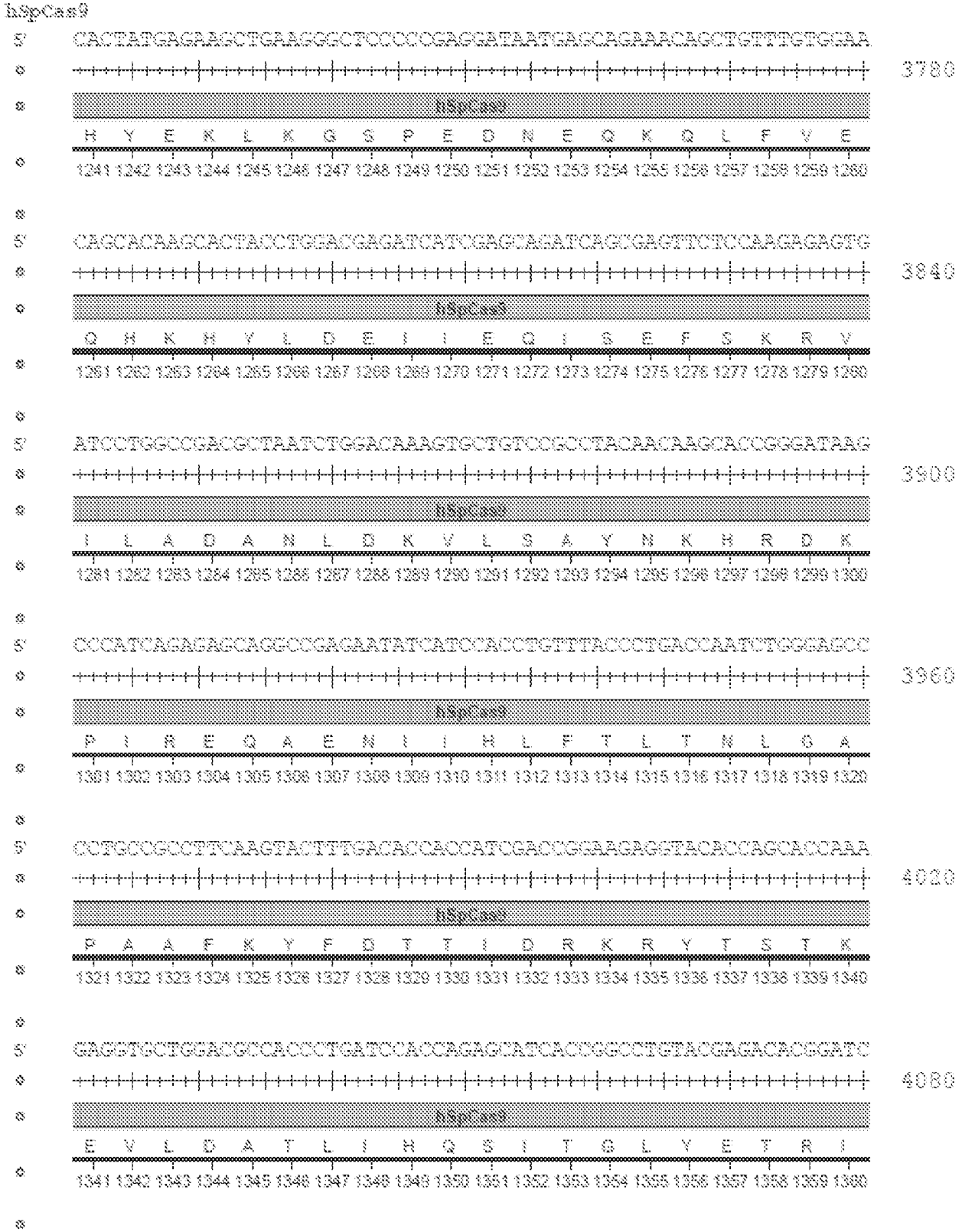

hSpCas9
5' CACTATGAGAAGCTGAAGGGCTCCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAA
3780
hSpCas9
H Y E K L K G S P E D N E Q K Q L F V E
1241 1242 1243 1244 1245 1246 1247 1248 1249 1250 1251 1252 1253 1254 1255 1256 1257 1258 1259 1260
5' CAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTG
3840
hSpCas9
Q H K H Y L D E I I E Q I S E F S K R V
1261 1262 1263 1264 1265 1266 1267 1268 1269 1270 1271 1272 1273 1274 1275 1276 1277 1278 1279 1280
5' ATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAG
3900
hSpCas9
I L A D A N L D K V L S A Y N K H R D K
1281 1282 1283 1284 1285 1286 1287 1288 1289 1290 1291 1292 1293 1294 1295 1296 1297 1298 1299 1300
5' CCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCC
3960
hSpCas9
P I R E Q A E N I I H L F T L T N L G A
1301 1302 1303 1304 1305 1306 1307 1308 1309 1310 1311 1312 1313 1314 1315 1316 1317 1318 1319 1320
5' CCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAA
4020
hSpCas9
P A A F K Y F D T T I D R K R Y T S T K
1321 1322 1323 1324 1325 1326 1327 1328 1329 1330 1331 1332 1333 1334 1335 1336 1337 1338 1339 1340
5' GAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATC
4080
hSpCas9
E V L D A T L I H Q S I T G L Y E T R I
1341 1342 1343 1344 1345 1346 1347 1348 1349 1350 1351 1352 1353 1354 1355 1356 1357 1358 1359 1360

FIG. 24L

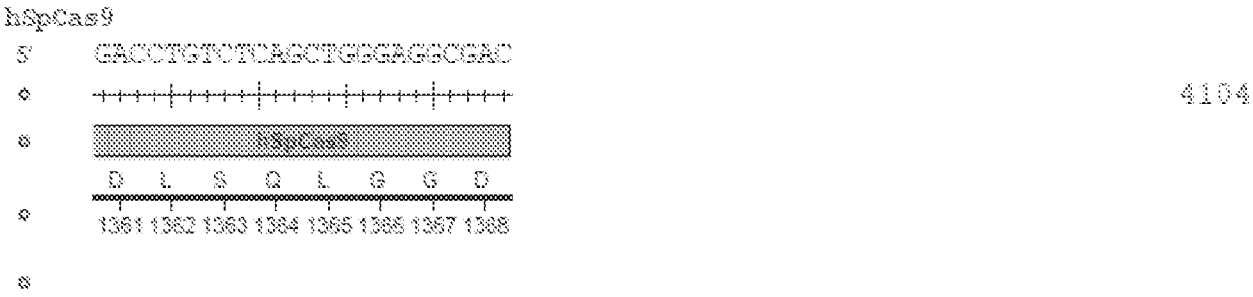

hSpCas9
5'
GACCTGTCTCAGCTGGGAGGCGAC
4104
D L S Q L G G D
1361 1362 1363 1364 1365 1366 1367 1368

FIG. 24M

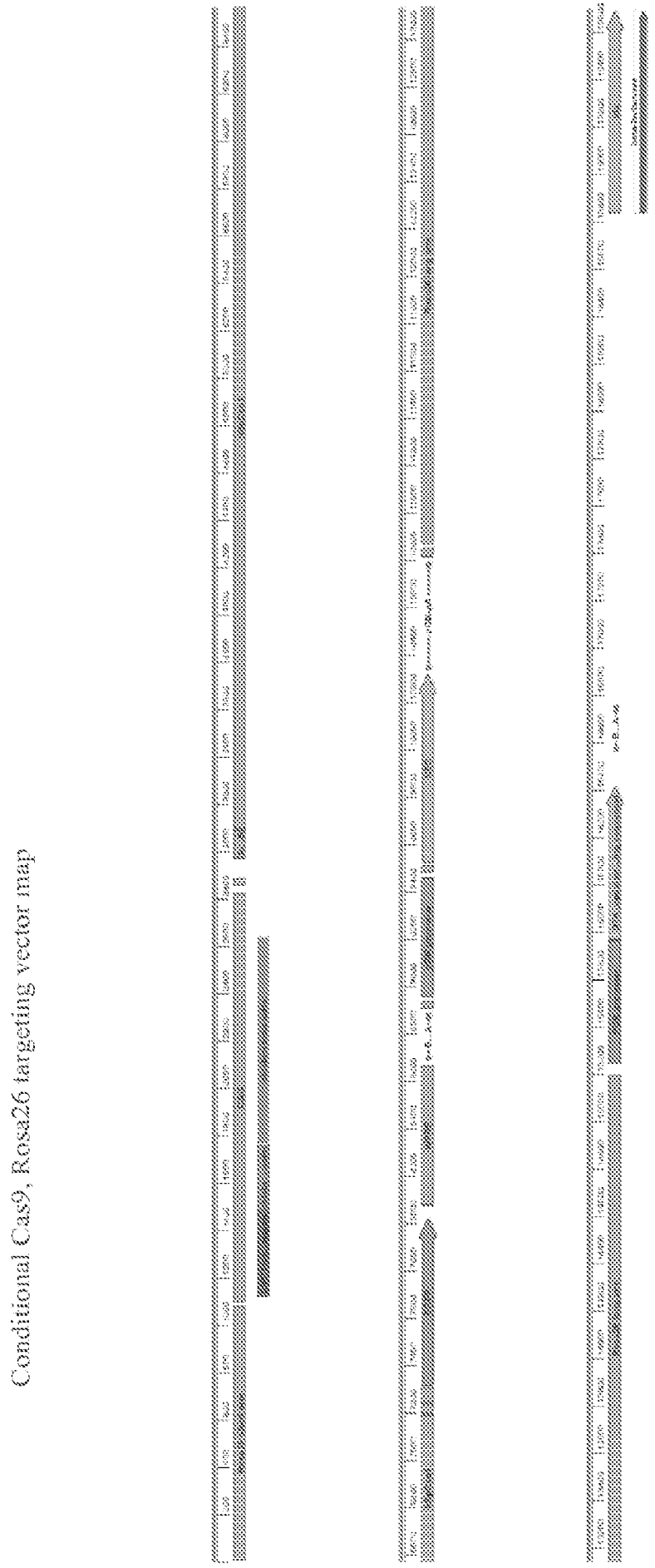


FIG. 25A

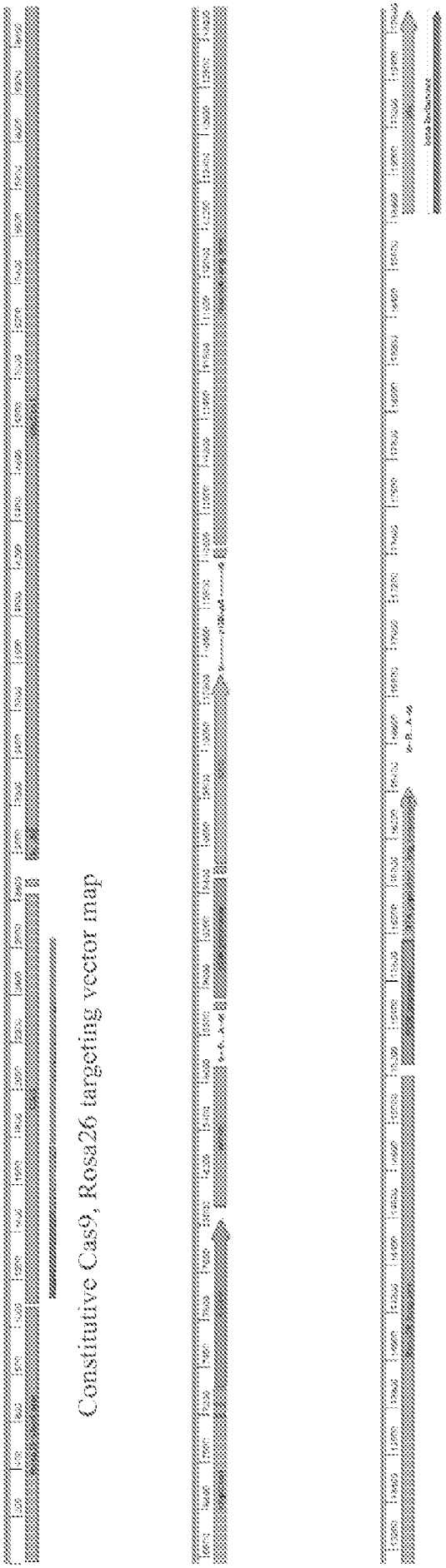


FIG. 25B

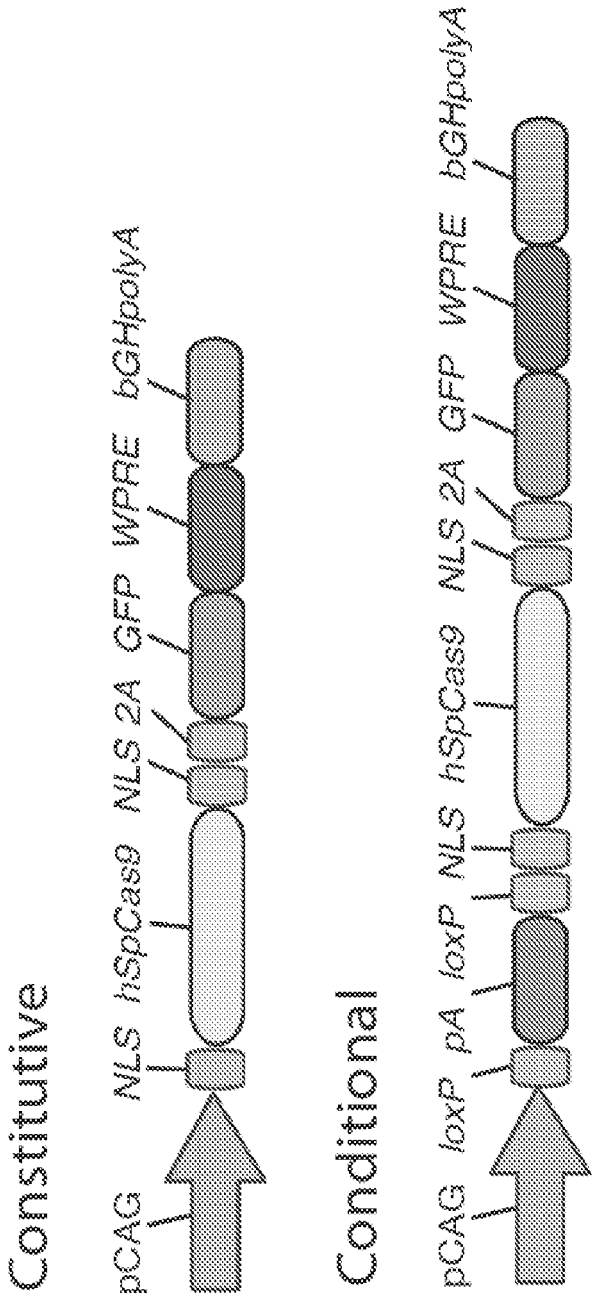
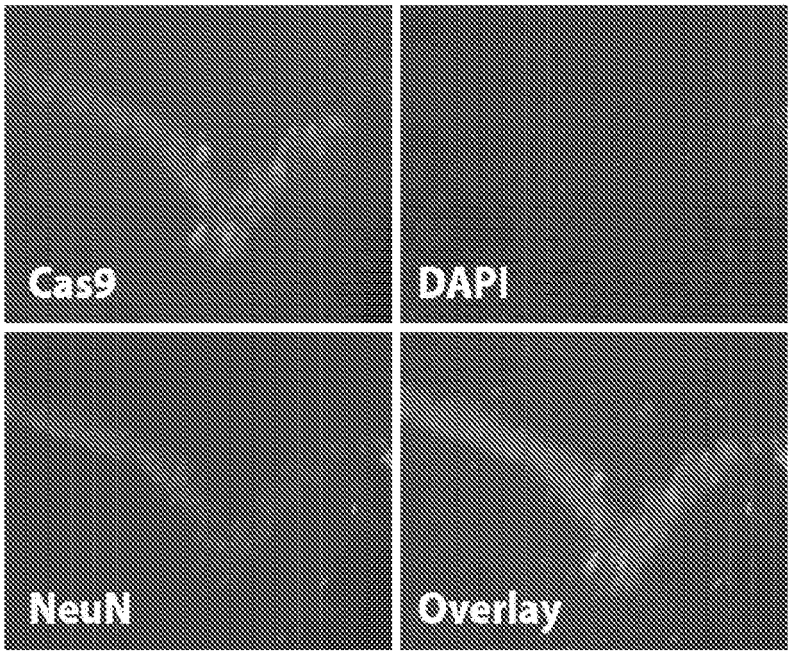

Constitutive
pCAG
NLS
hSpCas9
NLS
2A
GFP
WPRE
bGHpolyA
Conditional
pCAG
loxP
pA
loxP
NLS
hSpCas9
NLS
2A
GFP
WPRE
bGHpolyA

FIG. 26

Cas9 Expression in Mouse Hippocampus (AAV)



Cas9 Expression in Mouse Cortex (AAV)

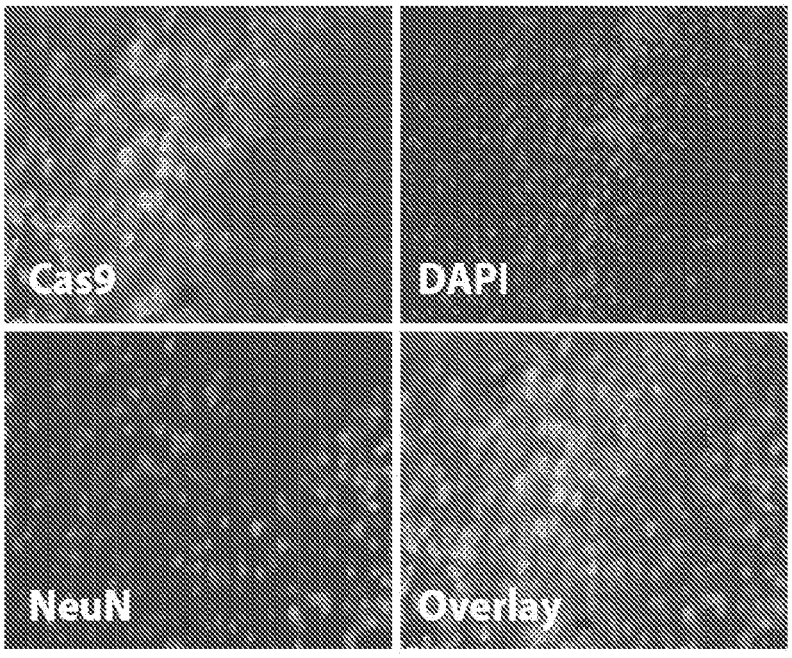


FIG. 27

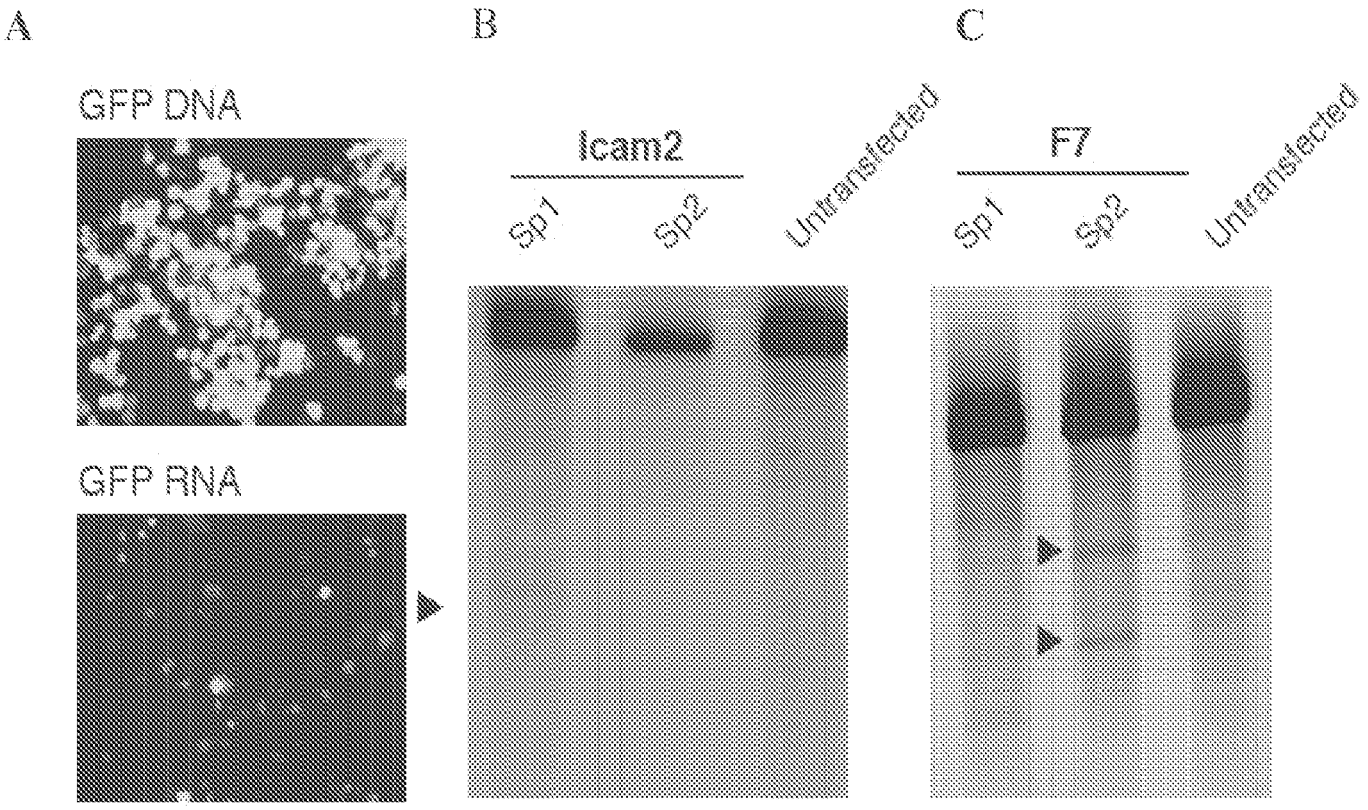

A
B
C
GFP DNA
GFP RNA
Icam2
F7
Sp1
Sp2
Untransfected
Sp1
Sp2
Untransfected

FIG. 28

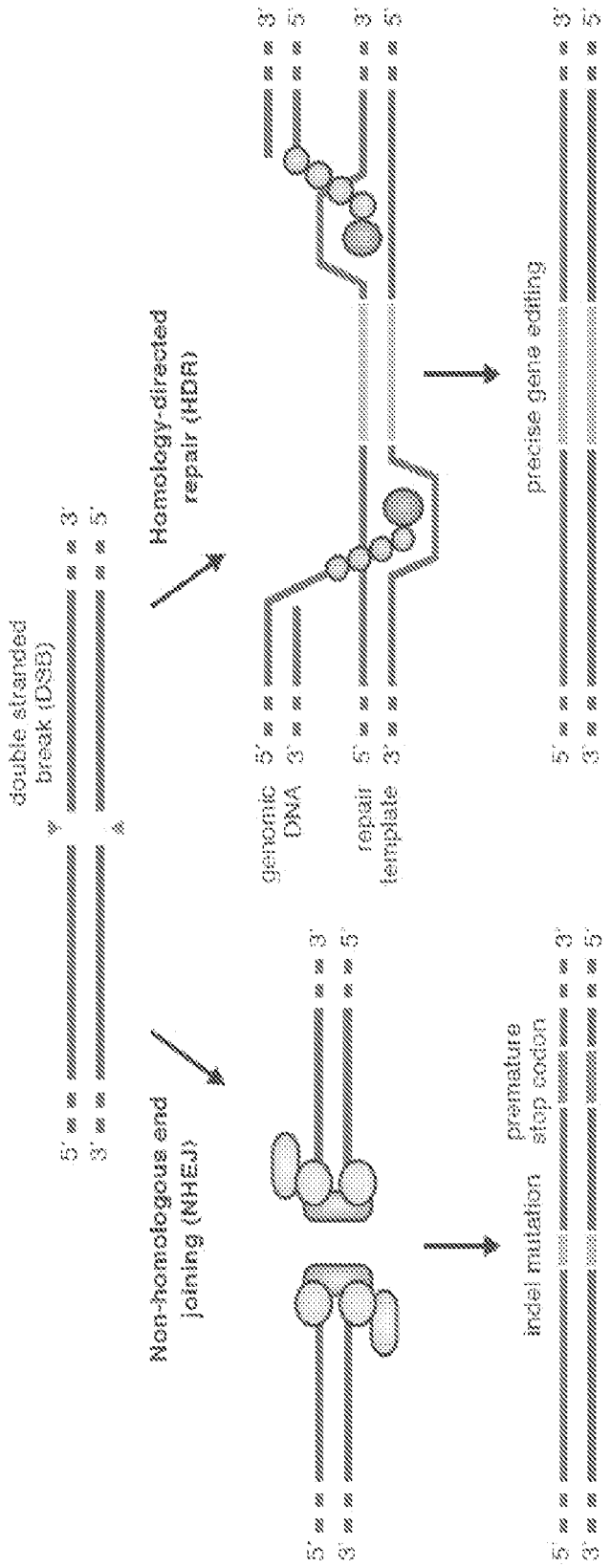

double stranded break (DSB)
5'
3'
3'
5'
Homology-directed repair (HDR)
genomic DNA
repair template
precise gene editing
Non-homologous end joining (NHEJ)
indel mutation
premature stop codon

FIG. 29

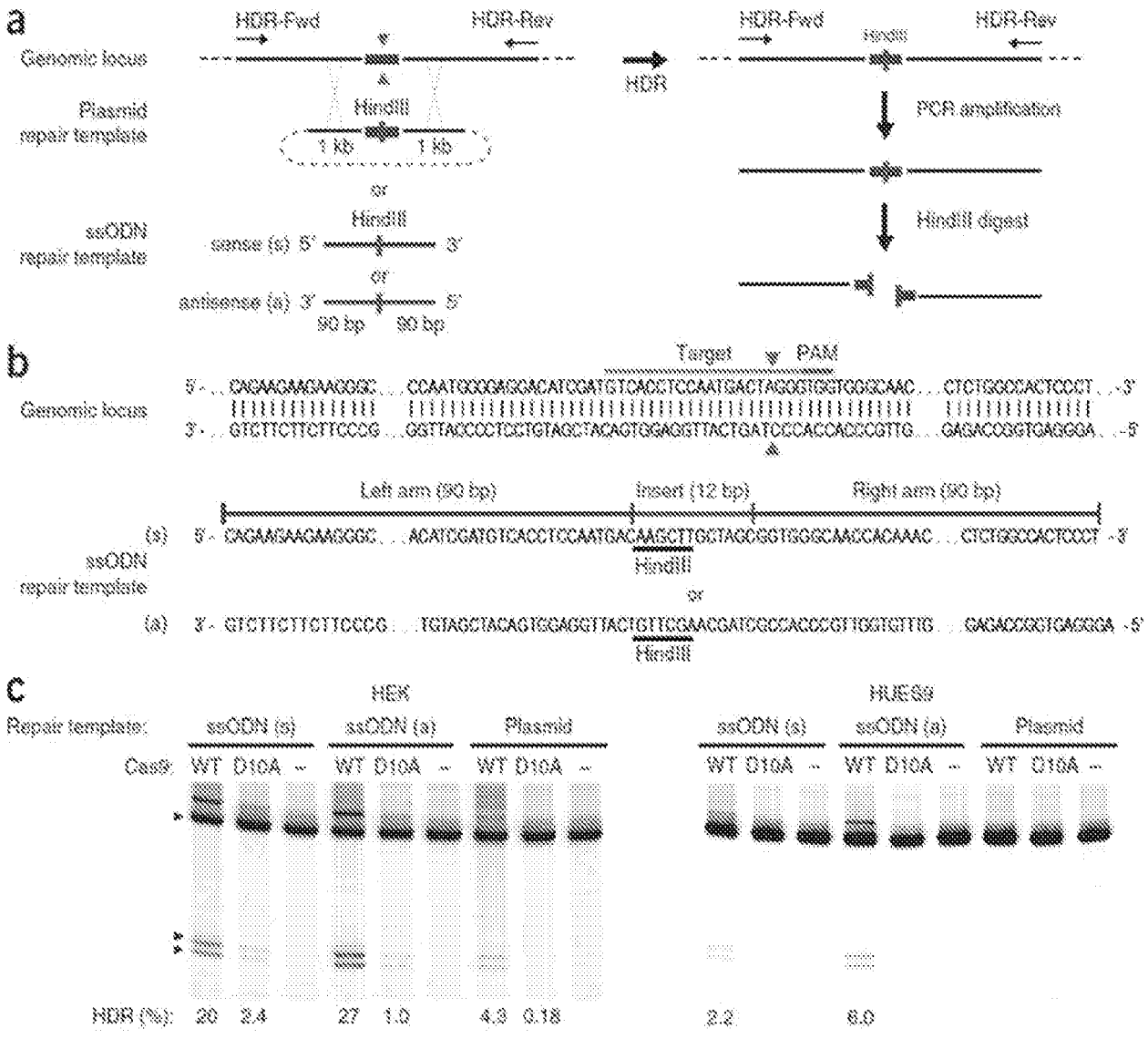

a
HDR-Fwd
HDR-Rev
Genomic locus
HDR
HindIII
PCR amplification
Plasmid repair template
1 kb
1 kb
or
ssODN repair template
sense (s) 5'
3'
antisense (a) 3'
5'
90 bp
90 bp
HindIII digest
b
Target
PAM
Genomic locus
Left arm (90 bp)
Insert (12 bp)
Right arm (90 bp)
(s)
(a)
ssODN repair template
HindIII
c
HEK
HUES9
Repair template:
ssODN (s)
ssODN (a)
Plasmid
Cas9:
WT
D10A
–
HDR (%):
20
2.4
27
1.0
4.3
0.18
2.2
6.0

FIG. 30A-C

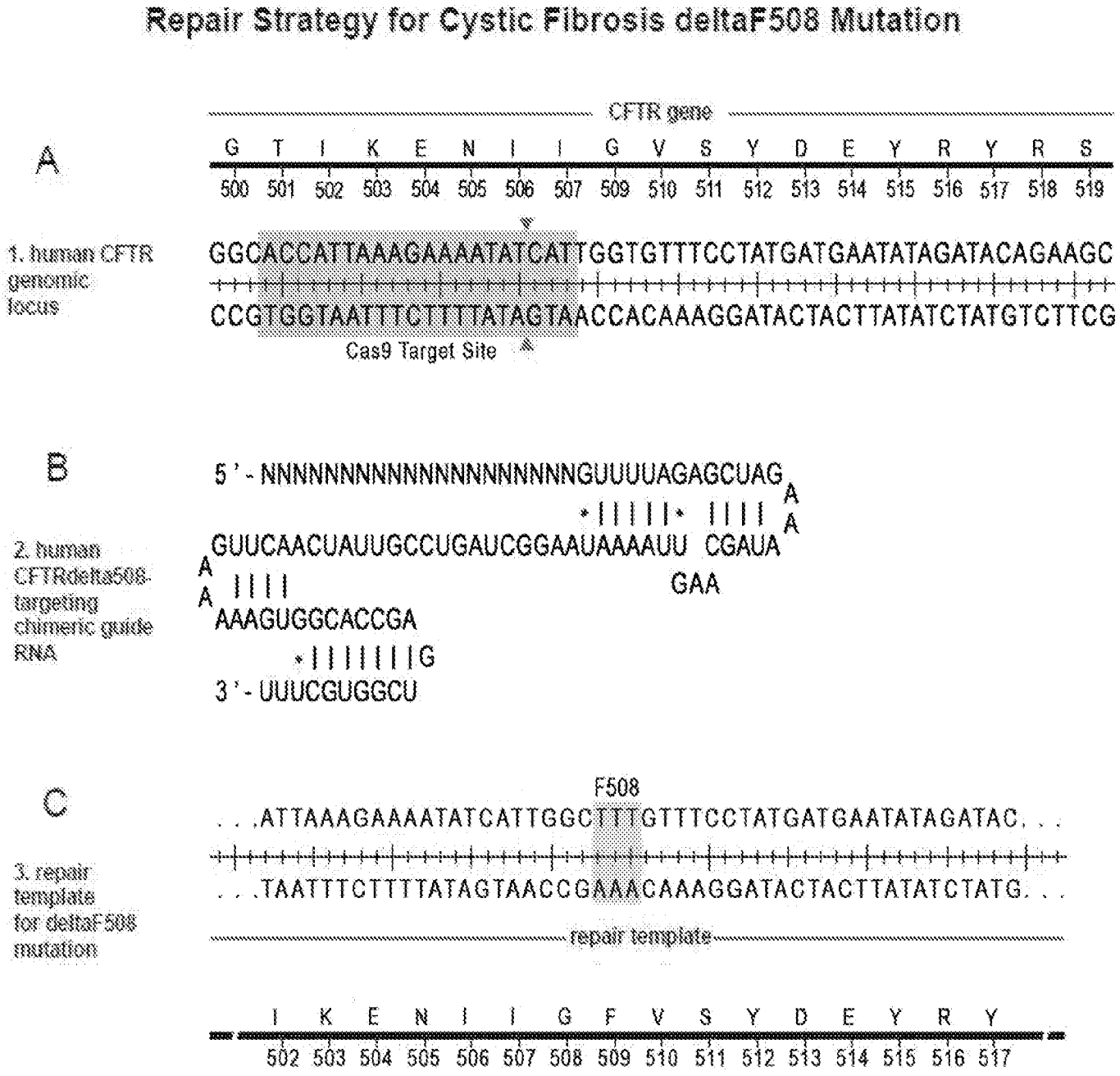

Repair Strategy for Cystic Fibrosis deltaF508 Mutation
A
CFTR gene
G T I K E N I I G V S Y D E Y R Y R S
500 501 502 503 504 505 506 507 509 510 511 512 513 514 515 516 517 518 519
1. human CFTR genomic locus
GGCACCATTAAAGAAAATATCATTGGTGTTTCCTATGATGAATATAGATACAGAAGC
CCGTGGTAATTTCTTTTATAGTAACCACAAAGGATACTACTTATATCTATGTCTTCG
Cas9 Target Site
B
5 ' - NNNNNNNNNNNNNNNNNNNGUUUUAGAGCUAG
A
GUUCAACUAUUGCCUGAUCGGAAUAAAAUU CGAUA
A
A
GAA
AAAGUGGCACCGA
3 ' - UUUCGUGGCU
2. human CFTRdelta508-targeting chimeric guide RNA
C
F508
. . .ATTAAAGAAAATATCATTGGCTTTGTTTCCTATGATGAATATAGATAC. . .
. . .TAATTTCTTTTATAGTAACCGAAACAAAGGATACTACTTATATCTATG. . .
repair template
I K E N I I G F V S Y D E Y R Y
502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517
3. repair template for deltaF508 mutation

FIG. 31

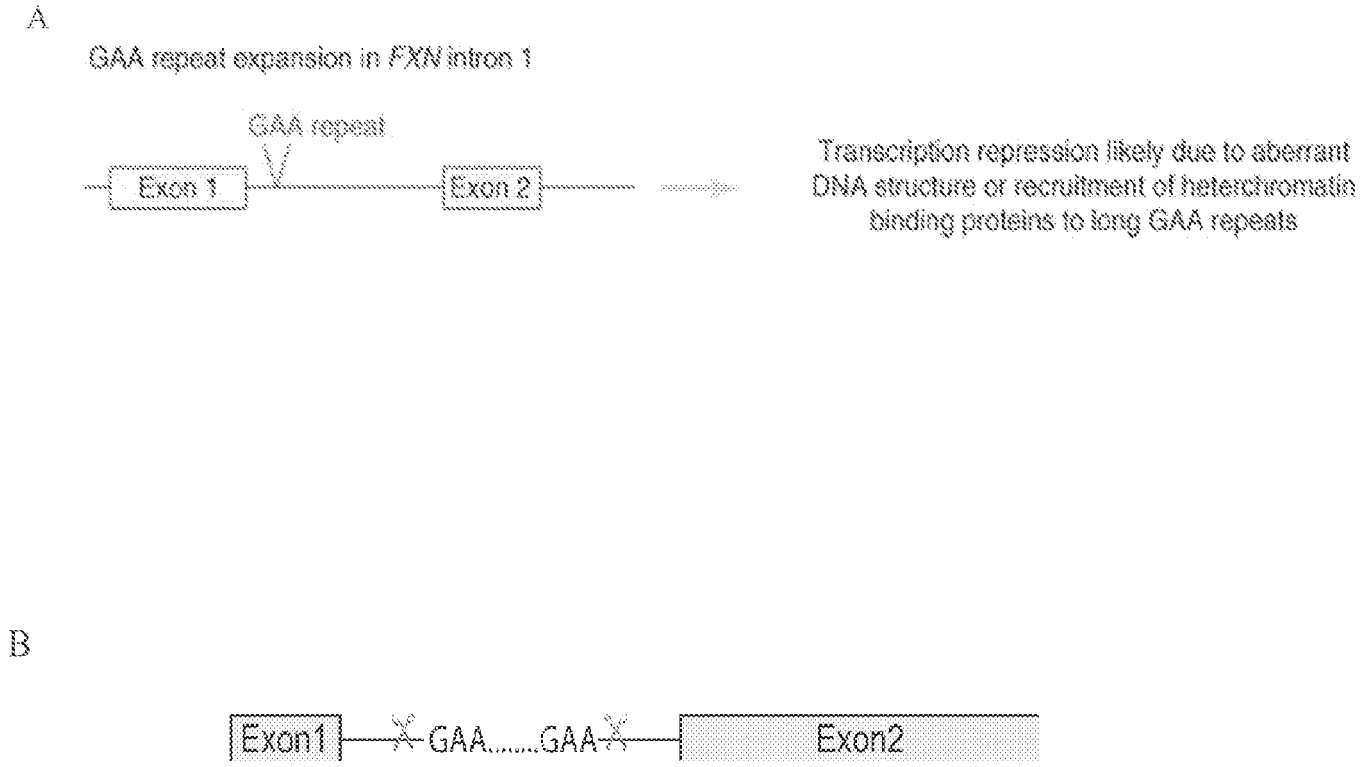

A
GAA repeat expansion in *FXN* intron 1
GAA repeat
Exon 1
Exon 2
Transcription repression likely due to aberrant DNA structure or recruitment of heterochromatin binding proteins to long GAA repeats
B
Exon1
GAA.....GAA
Exon2

FIG. 32

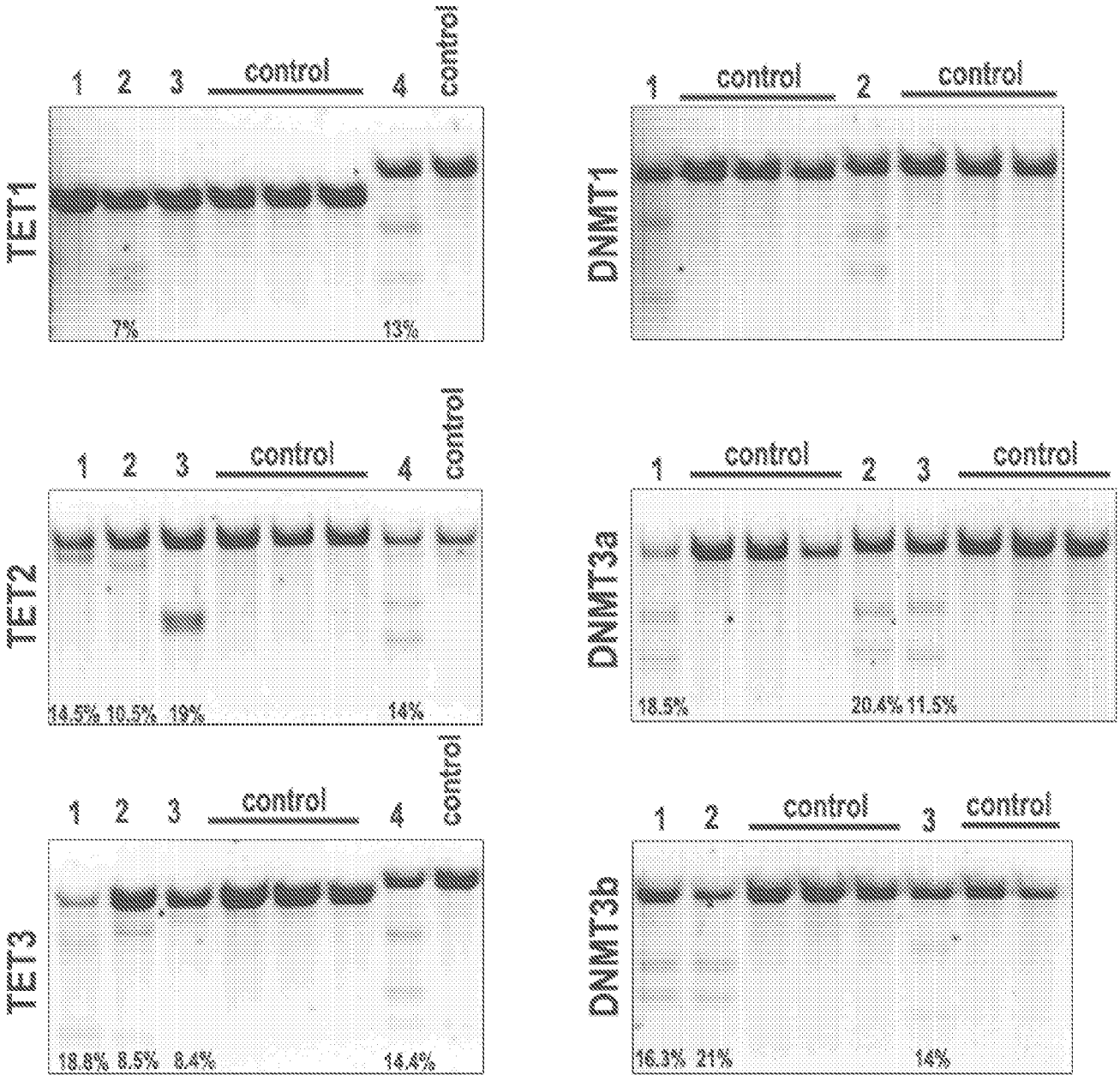

TET1
1 2 3 control 4 control
7%
13%
DNMT1
1 control 2 control
TET2
1 2 3 control 4 control
18.5% 10.5% 19%
14%
DNMT3a
1 control 2 3 control
18.5%
20.4% 11.5%
TET3
1 2 3 control 4 control
18.8% 8.5% 8.4%
14.4%
DNMT3b
1 2 control 3 control
16.3% 21%
14%

FIG. 33

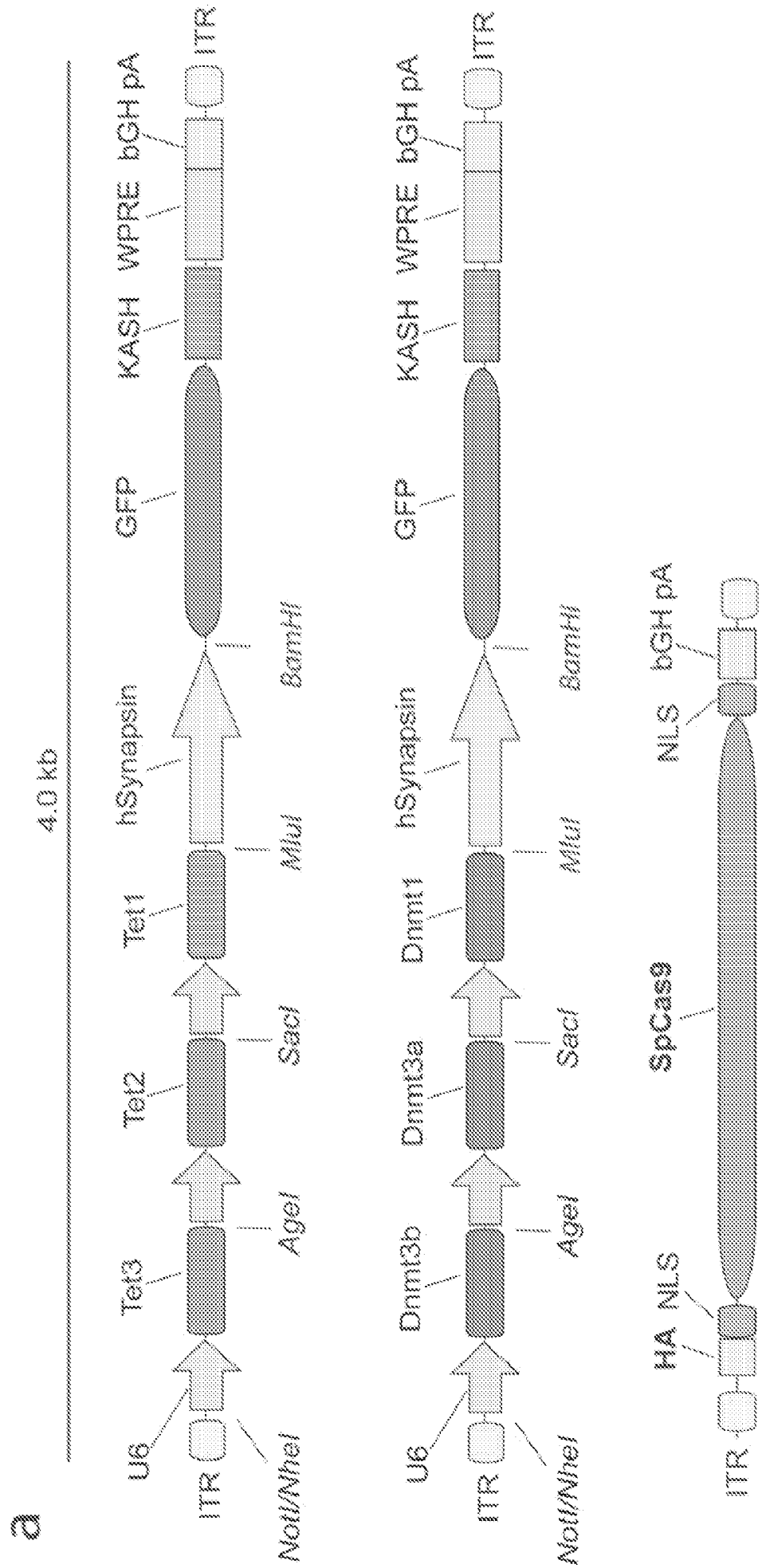

a
4.0 kb
U6
ITR
NotI/NheI
Tet3
AgeI
Tet2
SacI
Tet1
MluI
hSynapsin
GFP
KASH
WPRE
bGH pA
ITR
BamHI
Dnmt3b
Dnmt3a
Dnmt1
SpCas9
HA
NLS

FIG. 34

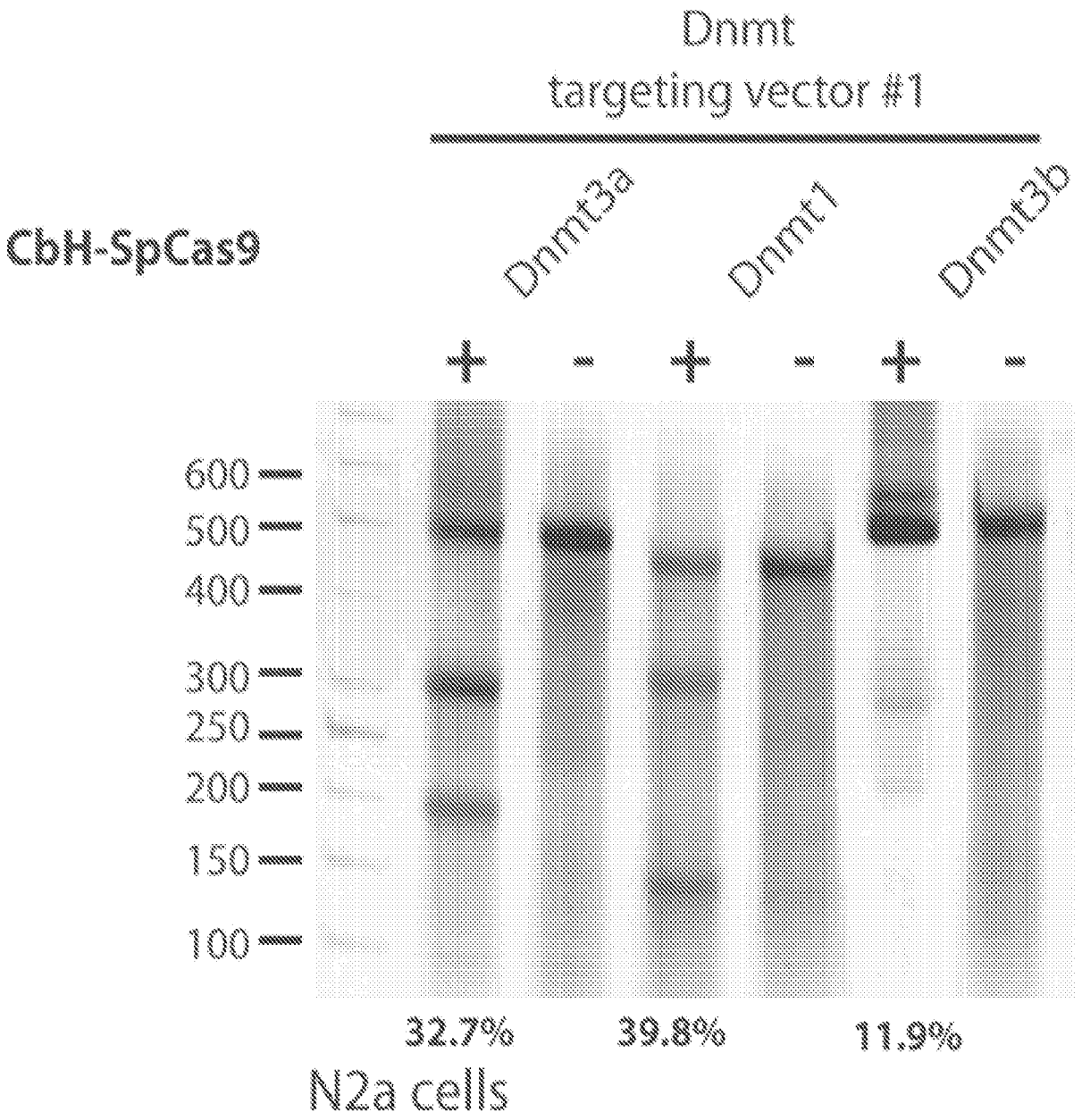

Dnmt
targeting vector #1
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+
-
+
-
+
-
600
500
400
300
250
200
150
100
32.7%
39.8%
11.9%
N2a cells

FIG. 35

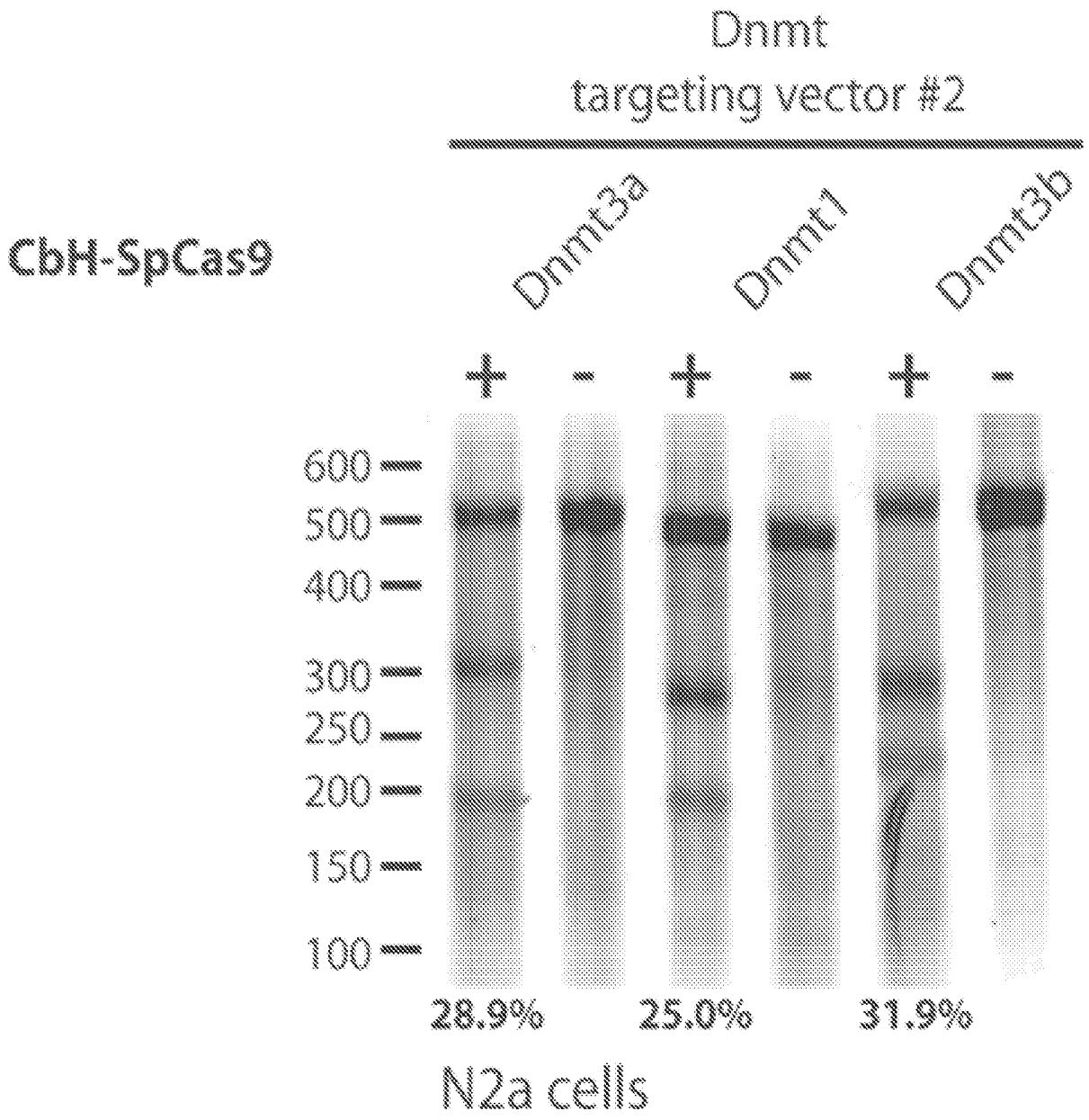

Dnmt
targeting vector #2
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+ - + - + -
600
500
400
300
250
200
150
100
28.9%
25.0%
31.9%
N2a cells

FIG. 36

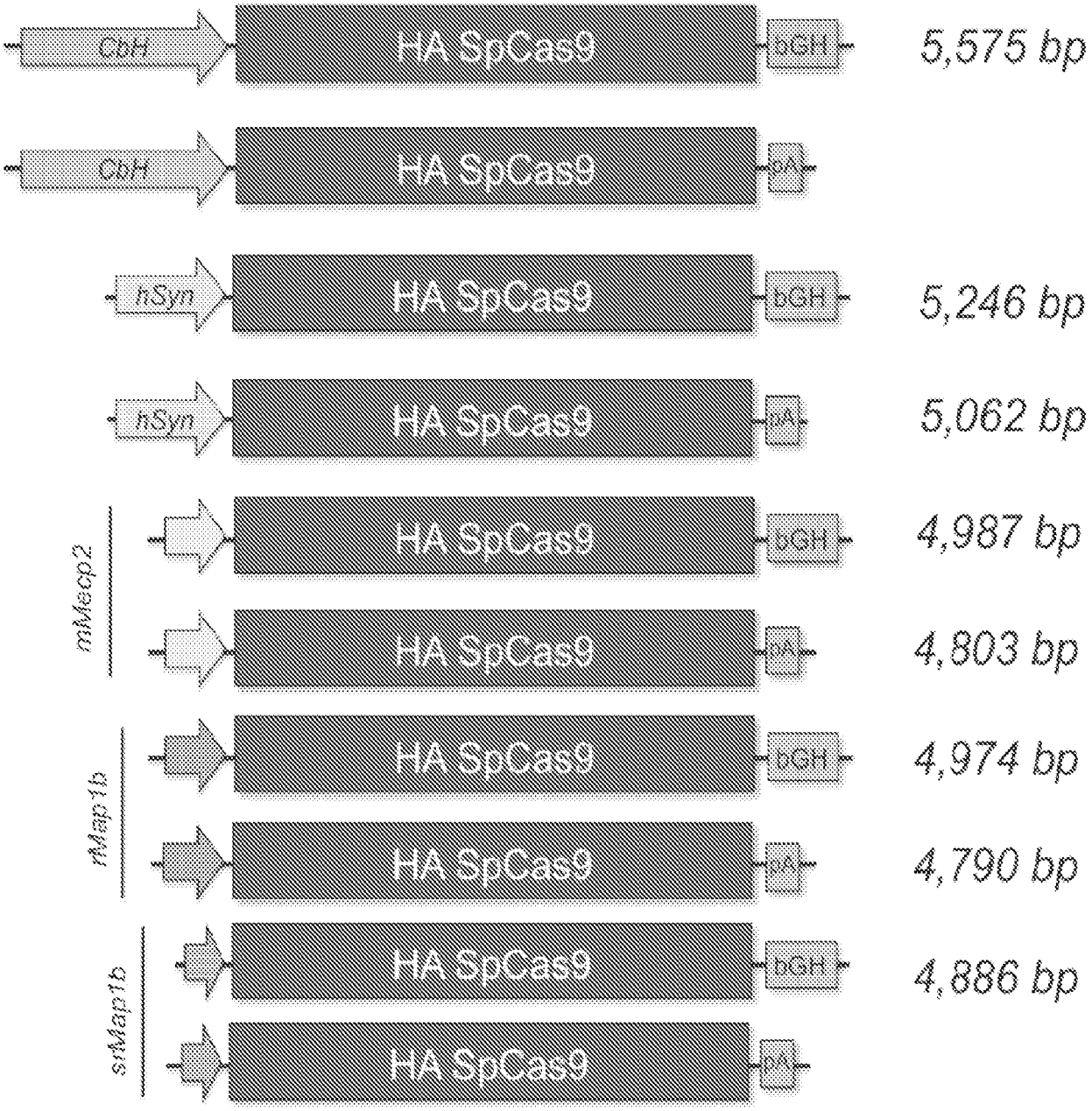

CbH
HA SpCas9
bGH
5,575 bp
CbH
HA SpCas9
hA
hSyn
HA SpCas9
bGH
5,246 bp
hSyn
HA SpCas9
hA
5,062 bp
mMecp2
HA SpCas9
bGH
4,987 bp
HA SpCas9
hA
4,803 bp
rMap1b
HA SpCas9
bGH
4,974 bp
HA SpCas9
hA
4,790 bp
srMap1b
HA SpCas9
bGH
4,886 bp
HA SpCas9
hA

FIG. 37

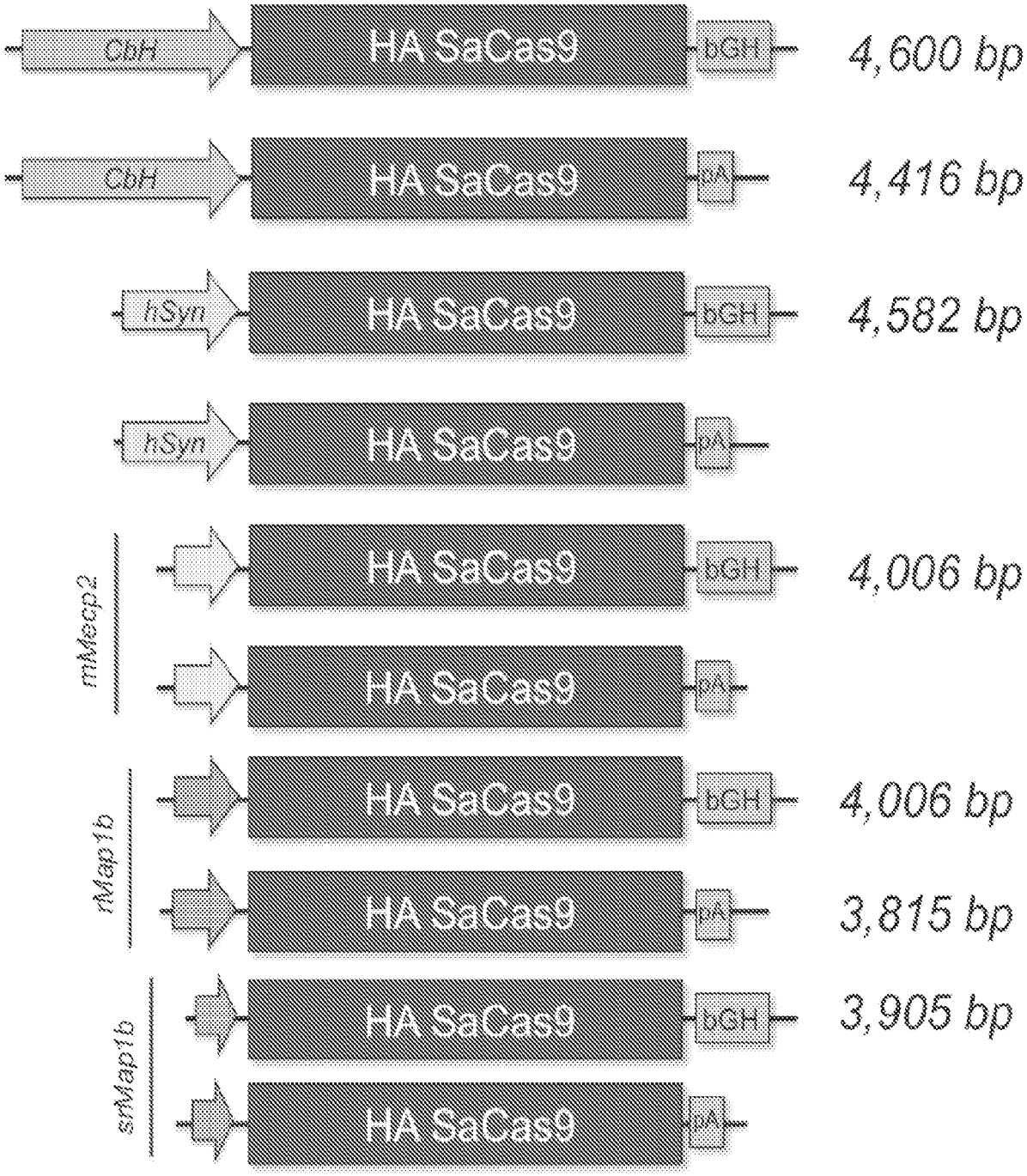

CbH
HA SaCas9
bGH
4,600 bp
CbH
HA SaCas9
pA
4,416 bp
hSyn
HA SaCas9
bGH
4,582 bp
hSyn
HA SaCas9
pA
rMecp2
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
rMap1b
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
3,815 bp
srMap1b
HA SaCas9
bGH
3,905 bp
HA SaCas9
pA

FIG. 38

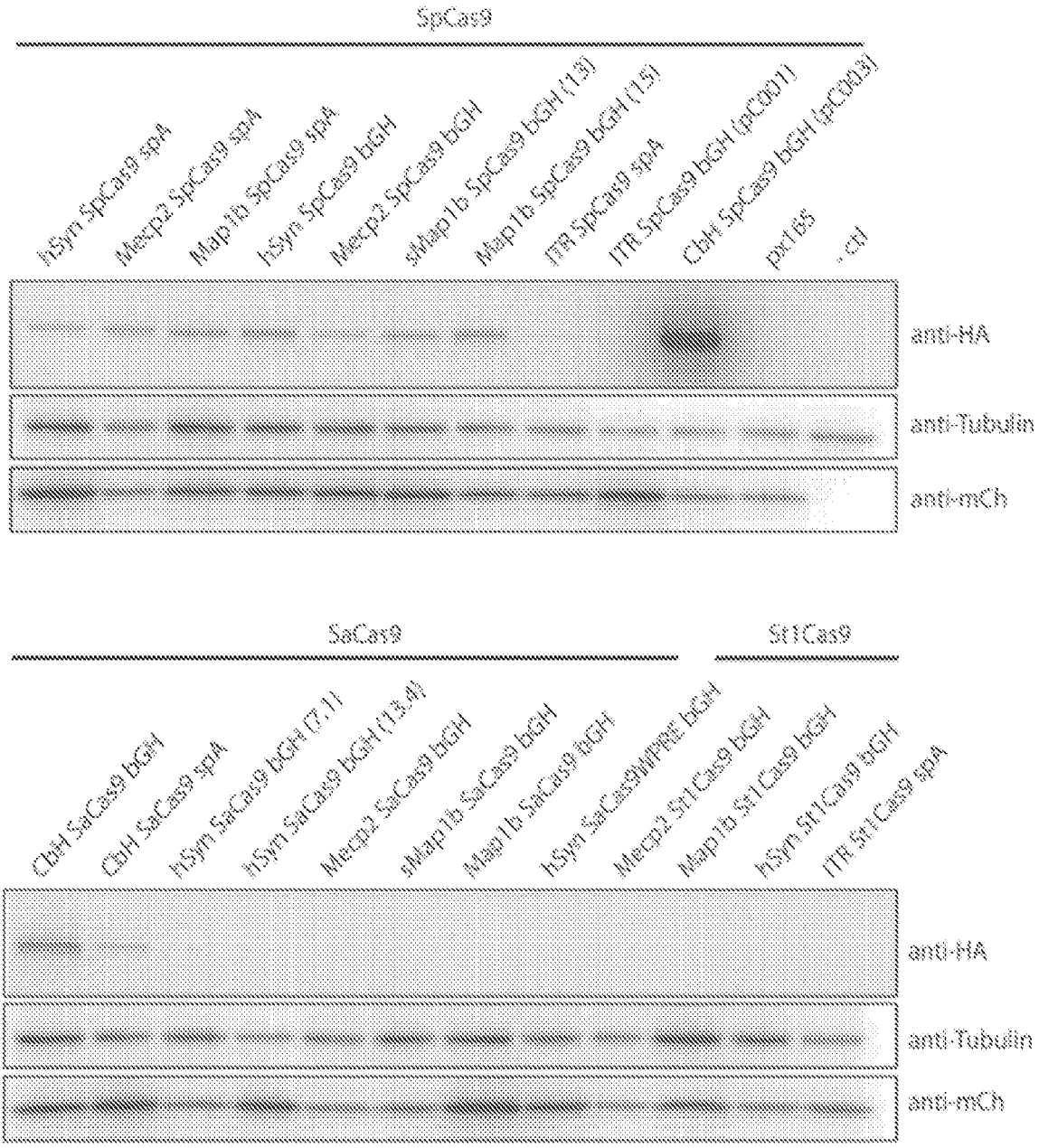

Expression of SpCas9 & SaCas9 in N2a cells
SpCas9
hSyn SpCas9 spA
Mecp2 SpCas9 spA
Map1b SpCas9 spA
hSyn SpCas9 bGH
Mecp2 SpCas9 bGH
shMap1b SpCas9 bGH (13)
Map1b SpCas9 bGH (15)
ITR SpCas9 spA
ITR SpCas9 bGH (pC001)
CBH SpCas9 bGH (pC003)
px165
anti-HA
anti-Tubulin
anti-mCh
SaCas9
St1Cas9
CBH SaCas9 bGH
CBH SaCas9 spA
hSyn SaCas9 bGH (7.1)
hSyn SaCas9 bGH (13.4)
Mecp2 SaCas9 bGH
shMap1b SaCas9 bGH
Map1b SaCas9 bGH
hSyn SaCas9WPRE bGH
Mecp2 St1Cas9 bGH
Map1b St1Cas9 bGH
hSyn St1Cas9 bGH
ITR St1Cas9 spA
anti-HA
anti-Tubulin
anti-mCh

FIG. 39

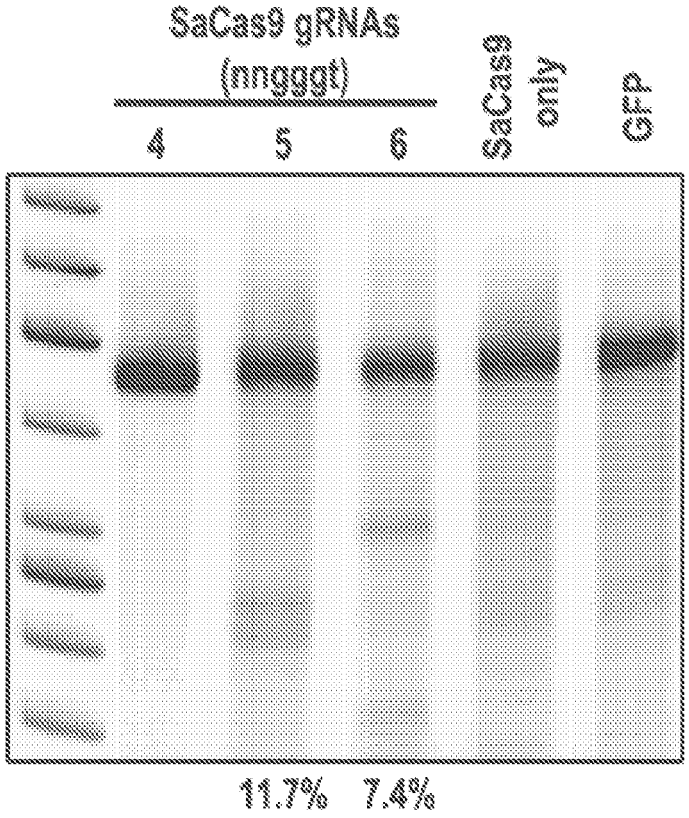


FIG. 40

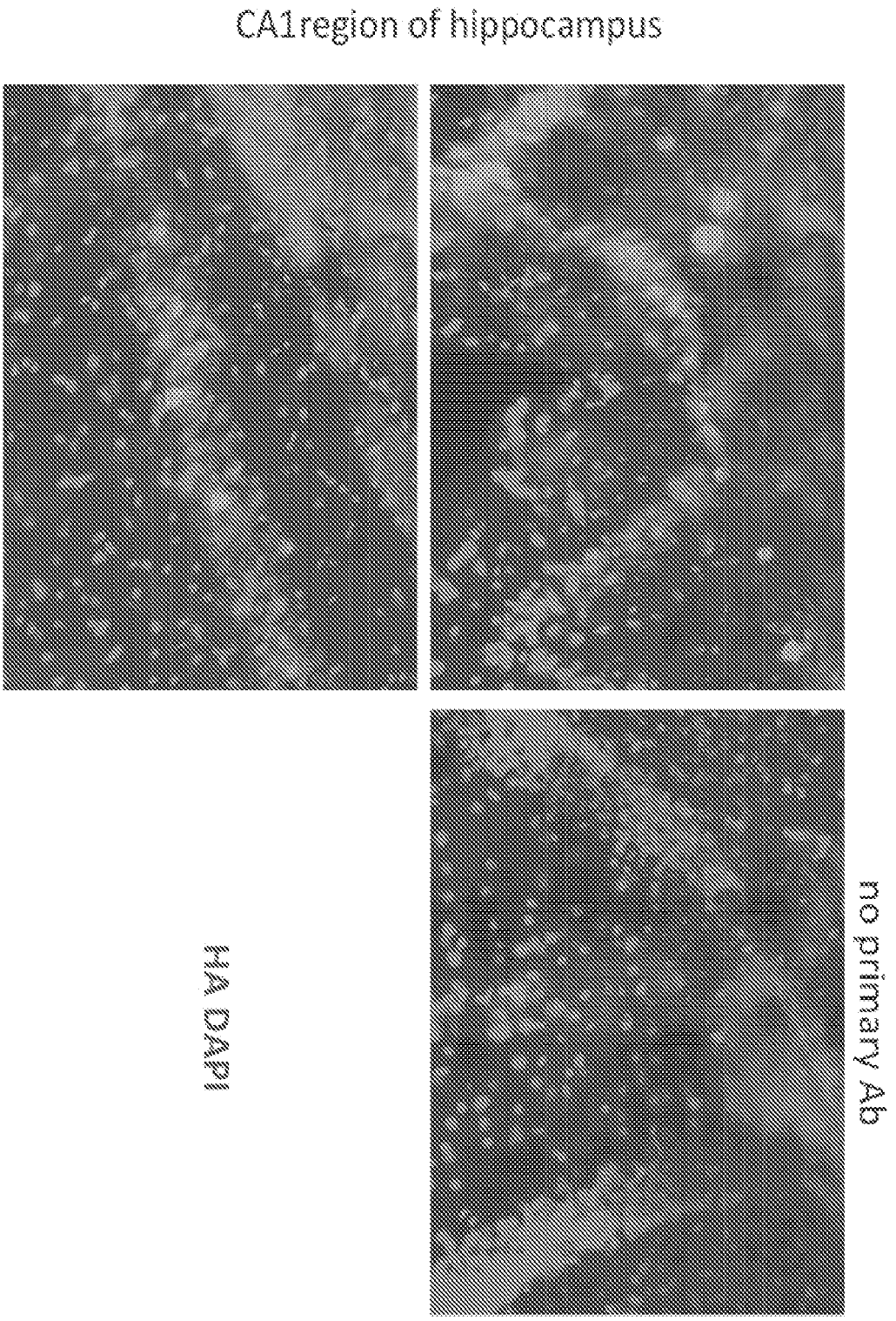


FIG. 41

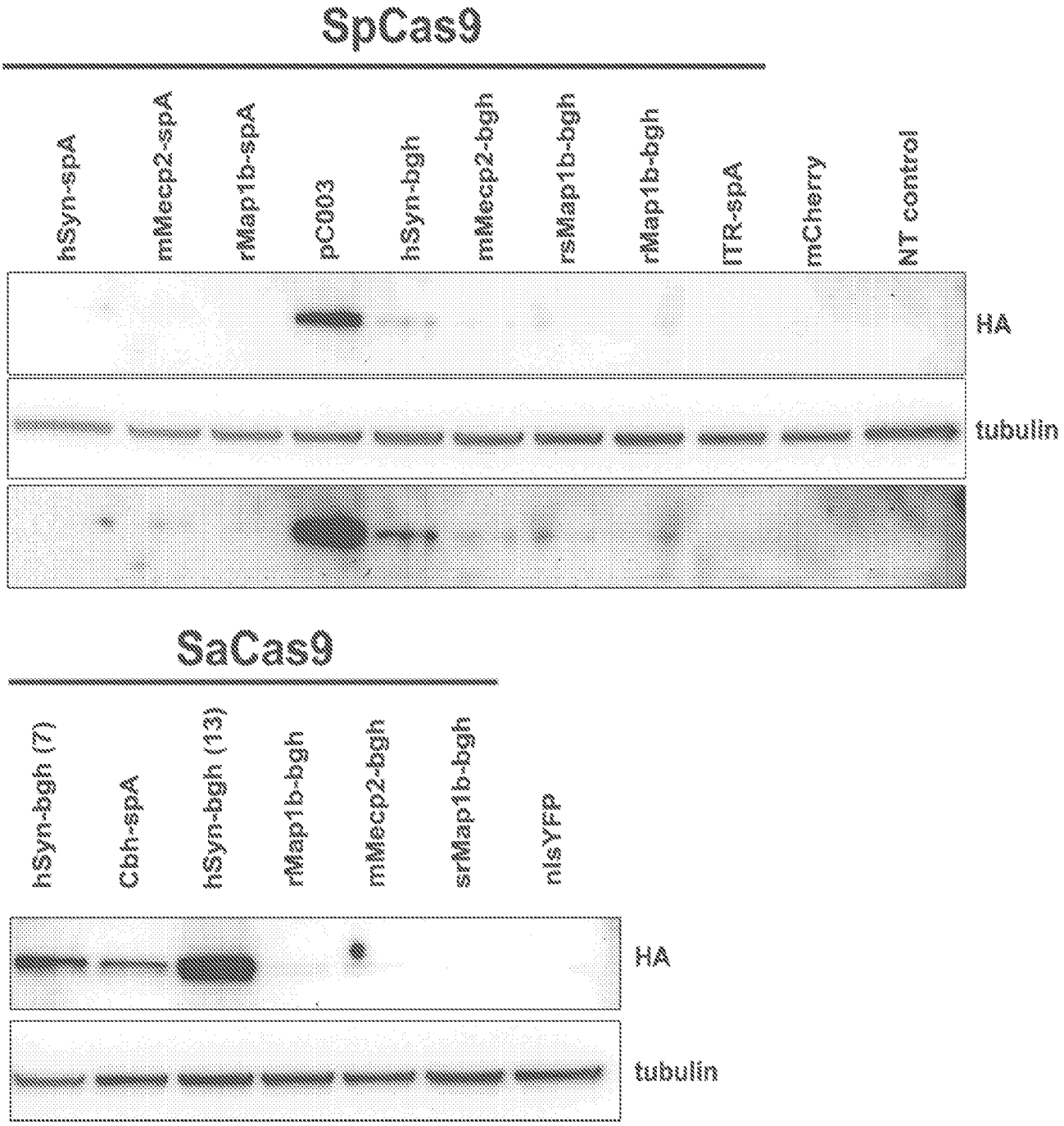

SpCas9
hSyn-spA
mMecp2-spA
rMap1b-spA
pC003
hSyn-bgh
mMecp2-bgh
rsMap1b-bgh
rMap1b-bgh
ITR-spA
mCherry
NT control
HA
tubulin
SaCas9
hSyn-bgh (7)
Cbh-spA
hSyn-bgh (13)
rMap1b-bgh
mMecp2-bgh
srMap1b-bgh
nlsYFP
HA
tubulin

FIG. 42

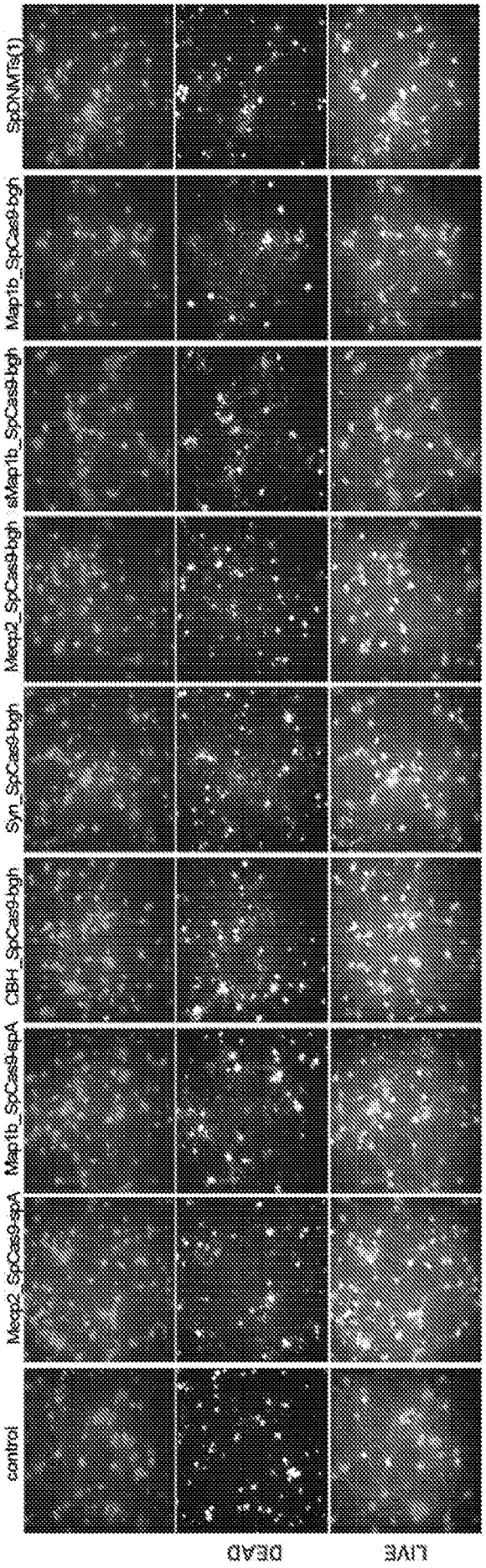

control
Mecp2_SpCas9-spA
Map1b_SpCas9-spA
CBH_SpCas9-bgh
Syn_SpCas9-bgh
Mecp2_SpCas9-bgh
sMap1b_SpCas9-bgh
Map1b_SpCas9-bgh
SpDNMTs(1)
DEAD
LIVE

FIG. 43

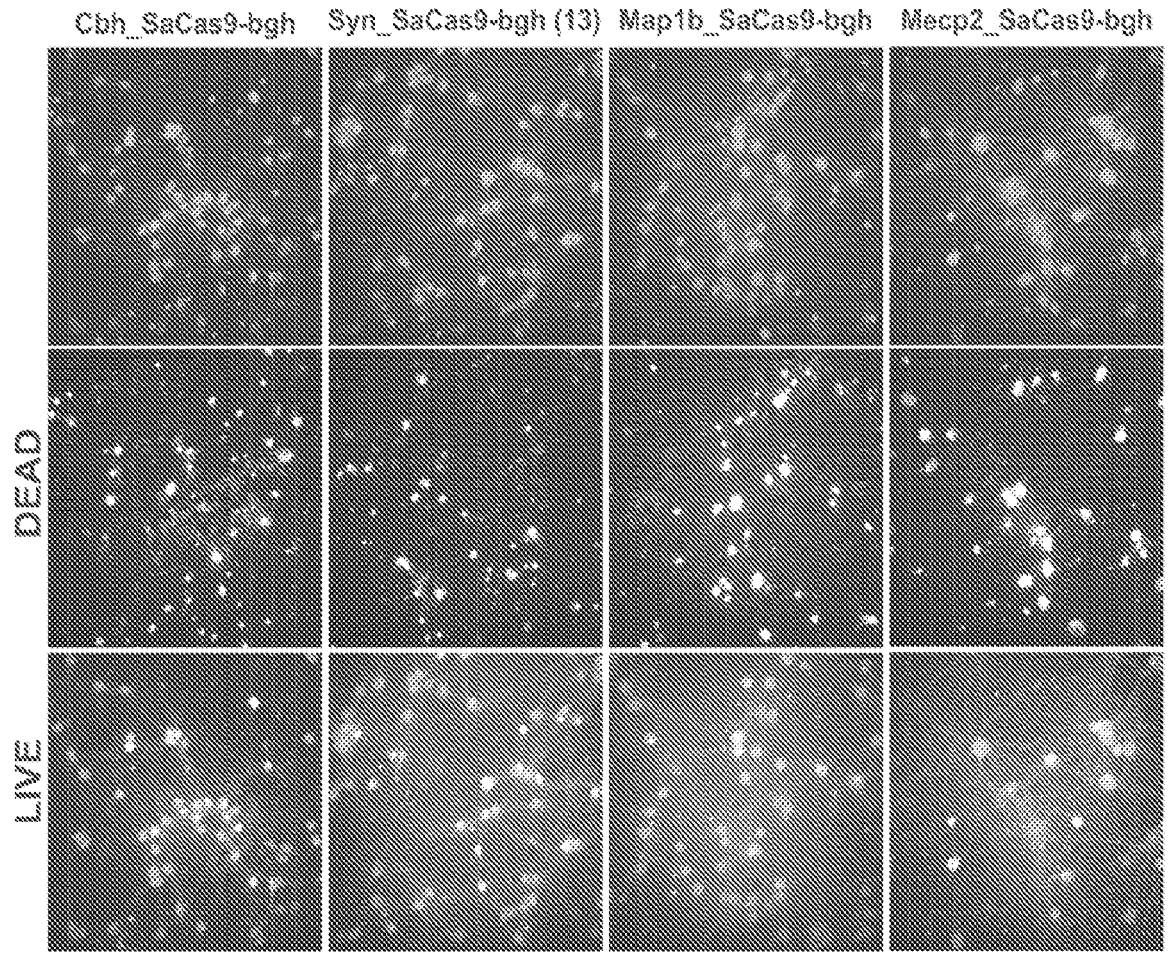

Cbh_SaCas9-bgh
Syn_SaCas9-bgh (13)
Map1b_SaCas9-bgh
Mecp2_SaCas9-bgh
DEAD
LIVE

FIG. 44

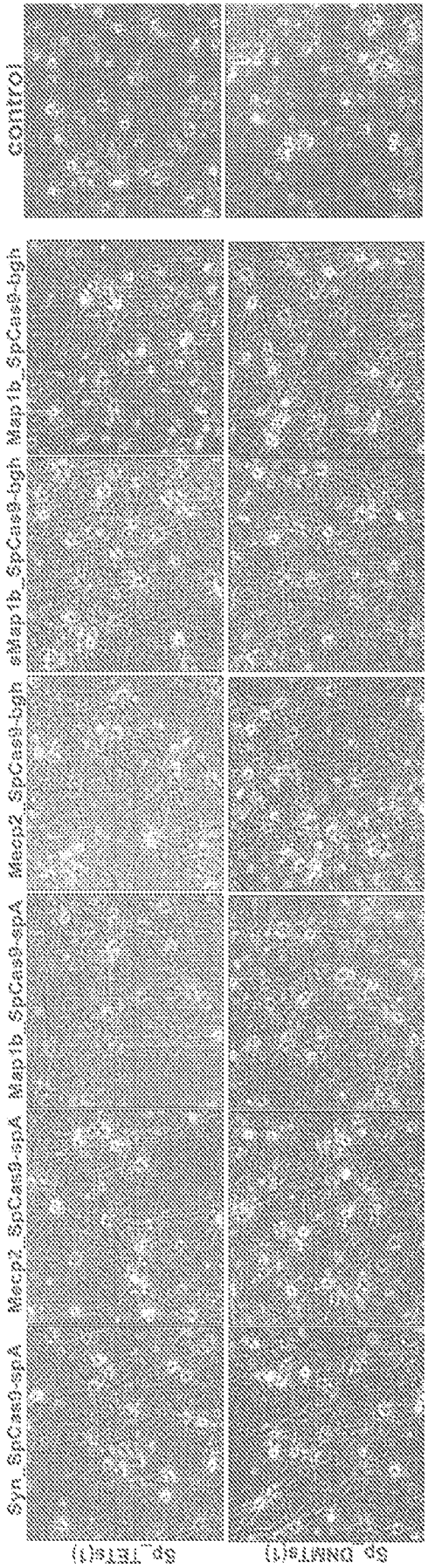

control
Syn_SpCas9-spA
Mecp2_SpCas9-spA
Map1b_SpCas9-spA
Mecp2_SpCas9-bgh
eMap1b_SpCas9-bgh
Map1b_SpCas9-bgh
Sp_DNMT3(1)
Sp_Tet3(1)

FIG. 45

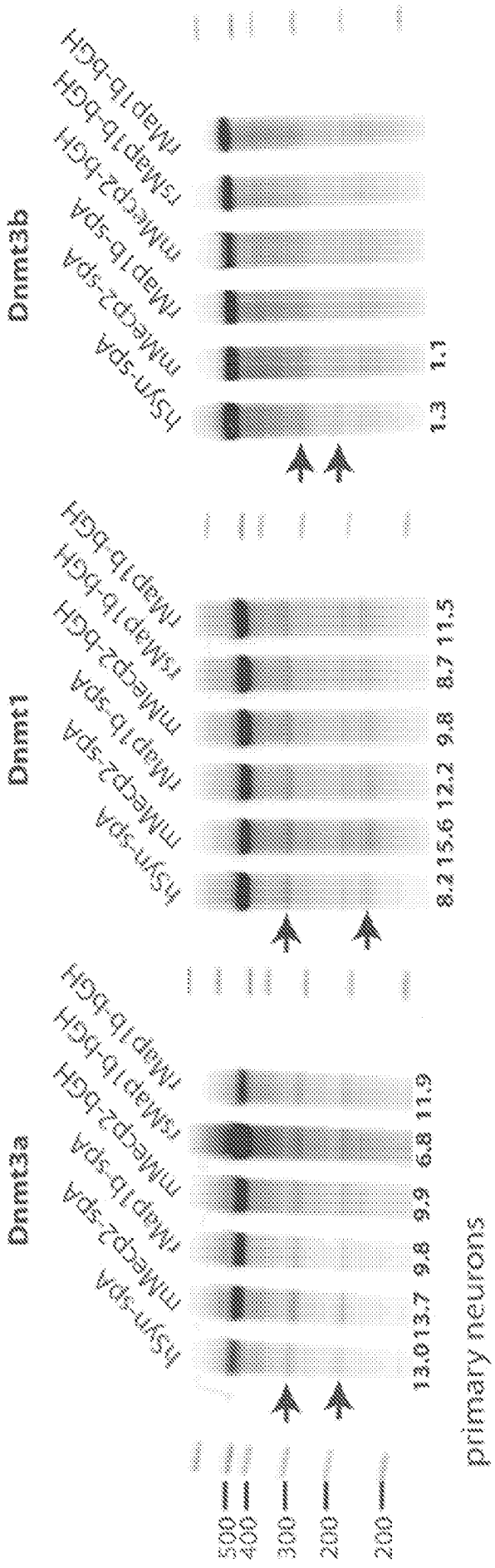

Dnmt3b
Dnmt1
Dnmt3a
hSyn-sPA
mMeCP2-sPA
mMap1b-sPA
rSMap1b-bGH
rSMeCP2-bGH
mMap1b-bGH
1.3
1.1
8.2
15.6
12.2
9.8
8.7
11.5
13.0
13.7
9.8
9.9
6.8
11.9
500
400
300
200
200
primary neurons

FIG. 46

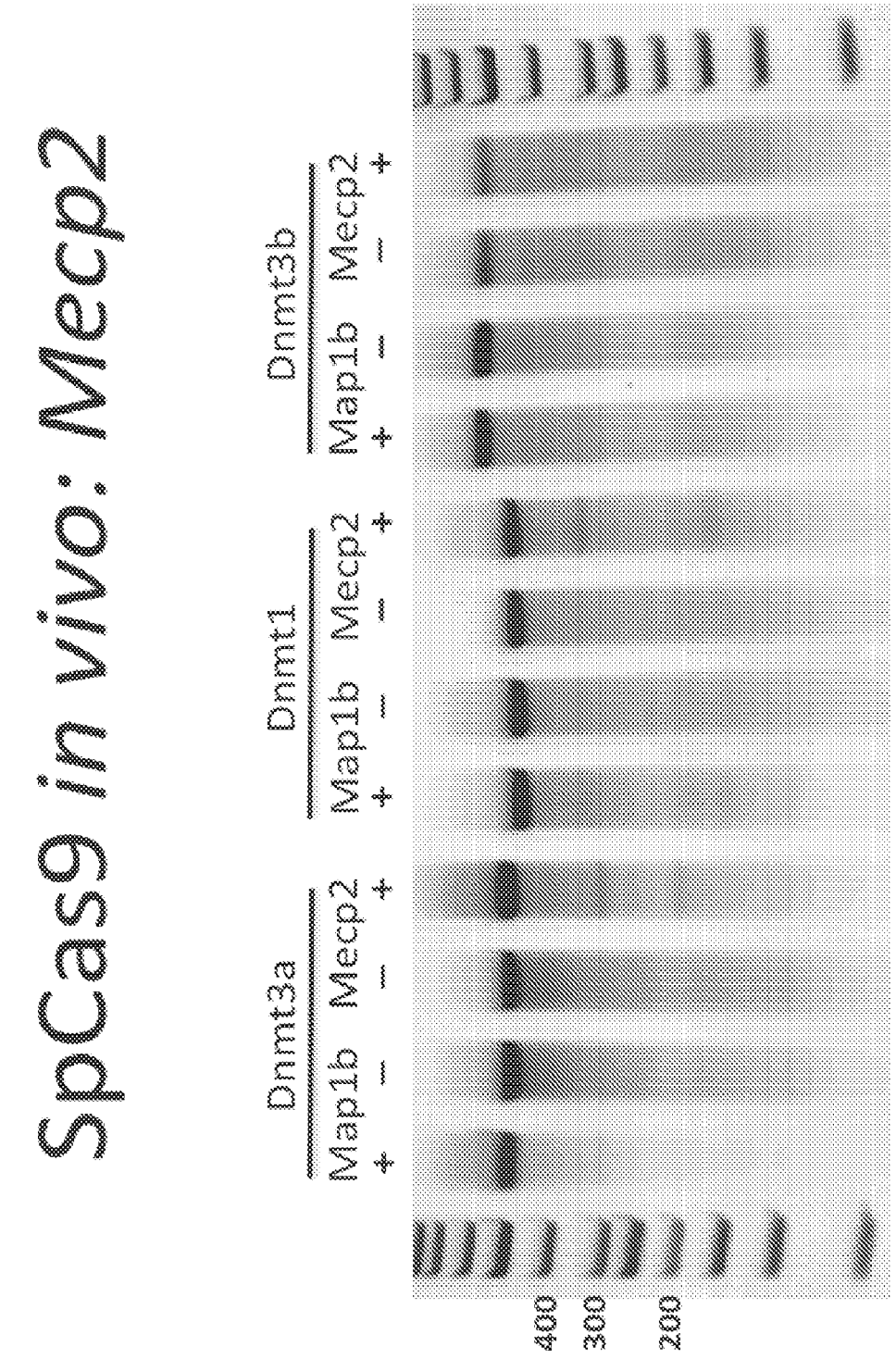

SpCas9 in vivo: Mecp2
Dnmt3a
Map1b + + –
Mecp2 – + +
Dnmt1
Map1b + + –
Mecp2 – + +
Dnmt3b
Map1b + + –
Mecp2 – + +
400
300
200
12.0%
17.0%

FIG. 47

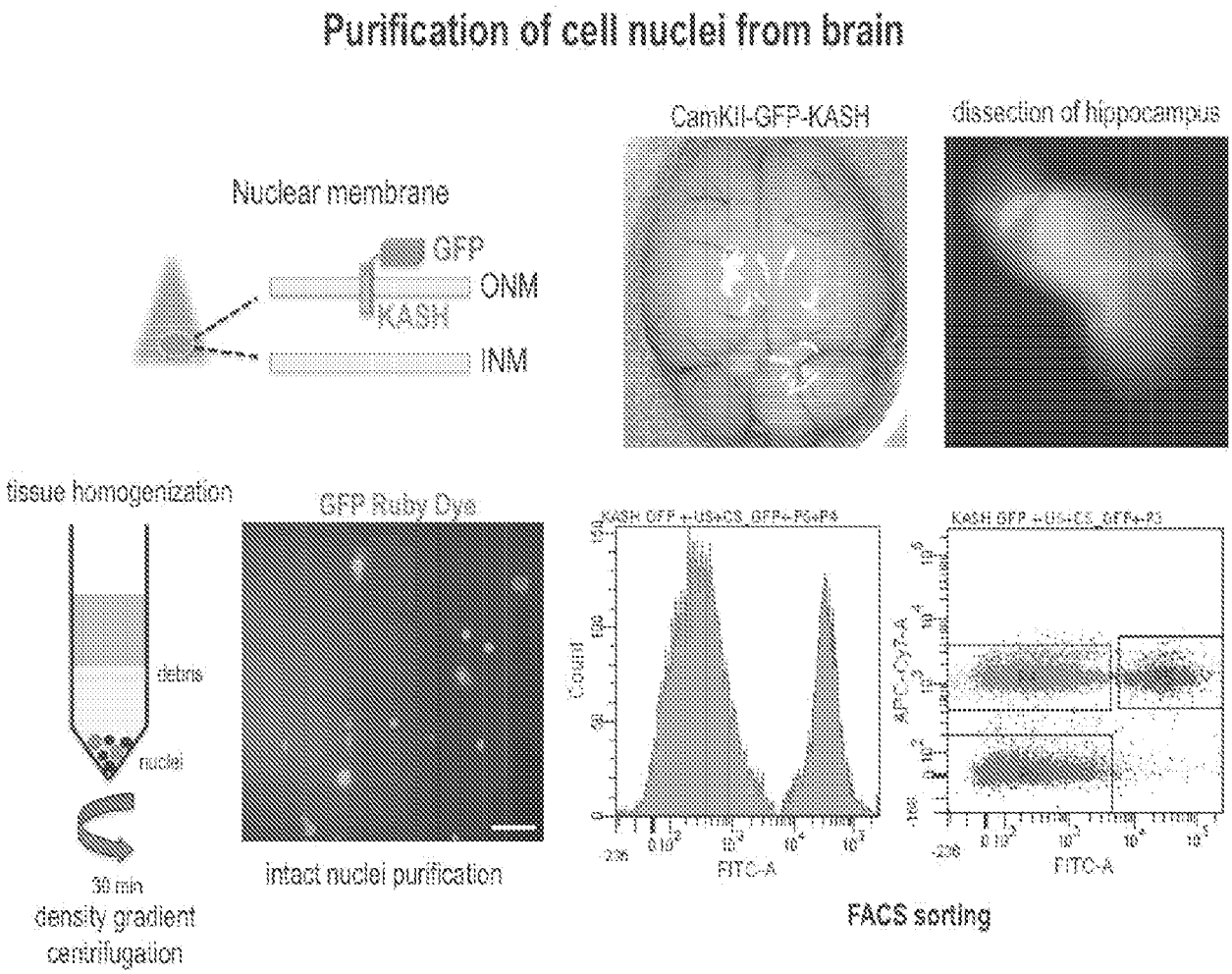

Purification of cell nuclei from brain
Nuclear membrane
GFP
ONM
KASH
INM
CamKII-GFP-KASH
dissection of hippocampus
tissue homogenization
detris
nuclei
30 min
density gradient
centrifugation
GFP Ruby Dye
intact nuclei purification
Count
FITC-A
APC-Cy7-A
FITC-A
FACS sorting

FIG. 48

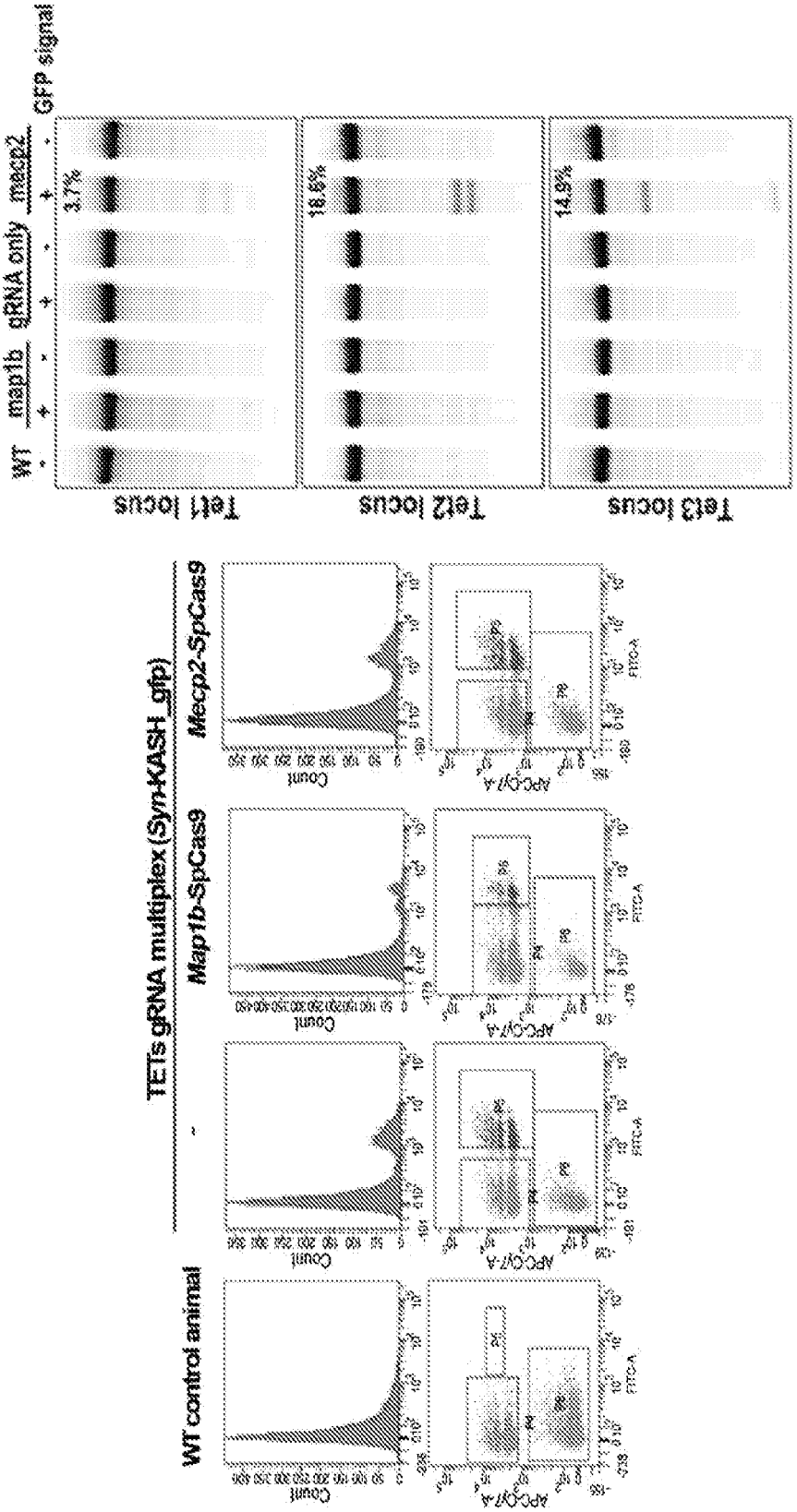

WT
map1b
gRNA only
mecp2
GFP signal
3.7%
18.6%
14.8%
Tet1 locus
Tet2 locus
Tet3 locus
TETs gRNA multiplex (Syn-KASH_gfp)
WT control animal
-
Map1b-SpCas9
Mecp2-SpCas9
Count
APC-Cy7-A
FITC-A

FIG. 49

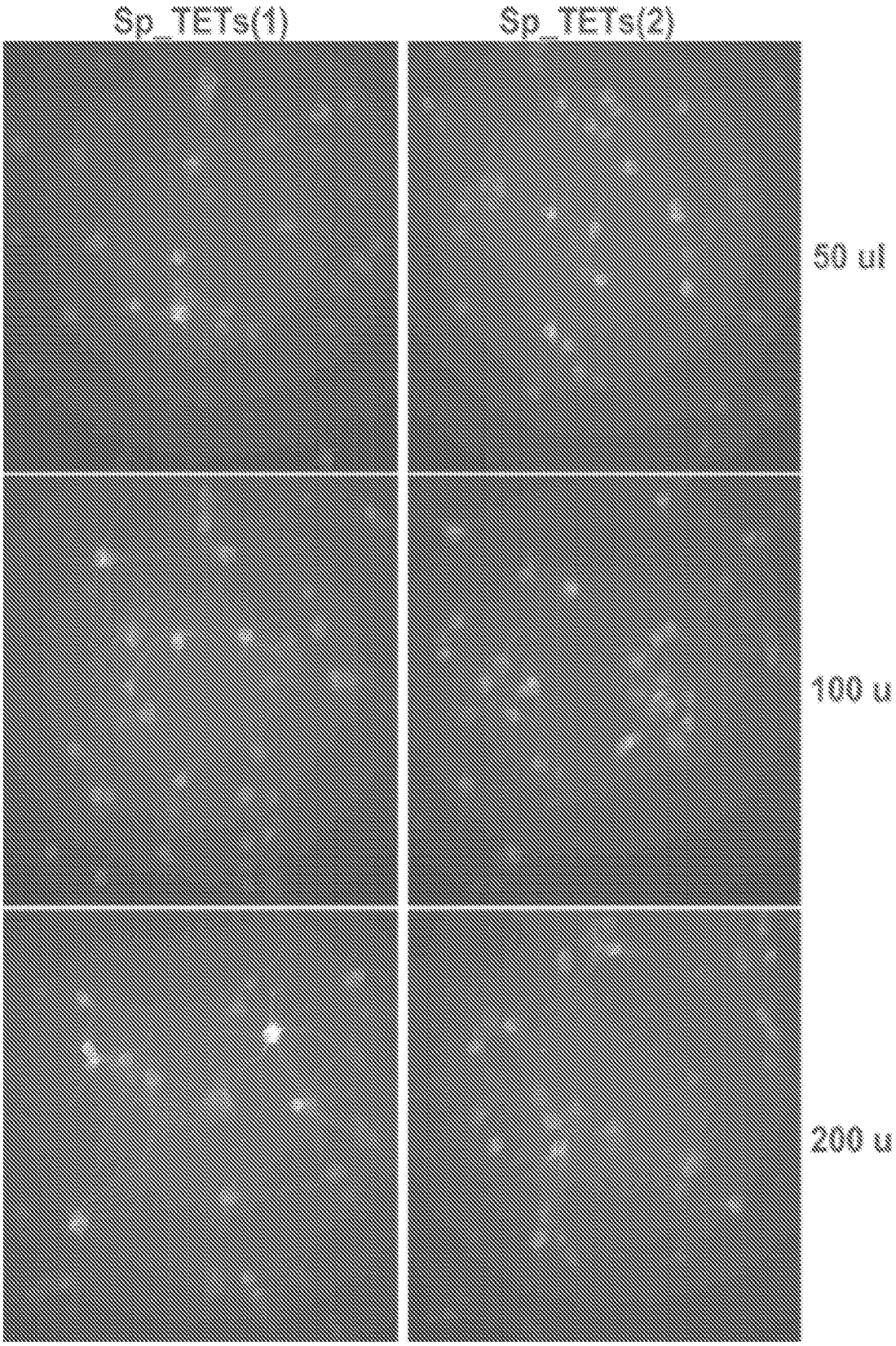


FIG. 50

	pattern	overhang
1	CCnNNNNNNNNNNNNNNNNnnnnnnnnnnnnNNNNNNNNNNNNNNNNnGG	38
2	CCnNNNNNNNNNNNNNNNNnnnnnnnnnNNNNNNNNNNNNNNNNnGG	34
3	CCnNNNNNNNNNNNNNNNNnnnnnNNNNNNNNNNNNNNNNNnGG	30
4	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	26
5	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	22
6	CCnNNNNNNNNNNNNNNNNNNNNNNNNnGG	18
7	CCnNNNNNNNNNNNNNNNNNNNNNnGG	14
8	CCnNNNNNNNNNNNNNNNNnGG	10
9	CCnNNNNNNNNNNNNNnGG	6
10	CCnNNNNNNNNNNnGG	4
11	CCnNNNNNNNNNnCG	3
12	CCnNNNNNNNNnGG	2
13	CCnNNNNNNNnGG	1
14	CCnNNNNNNnGG	0
15	CCnNNNNNnCG	1
16	CCnNNNNnGG	2
17	CCnNNNnGG	3
18	CCnnGG	4
19	CCGG	8
20	GGCC	10
21	GCNNCC	12
22	GCNNNCC	14
23	GCNNNNCC	16
24	GCNNNNNNNCC	20

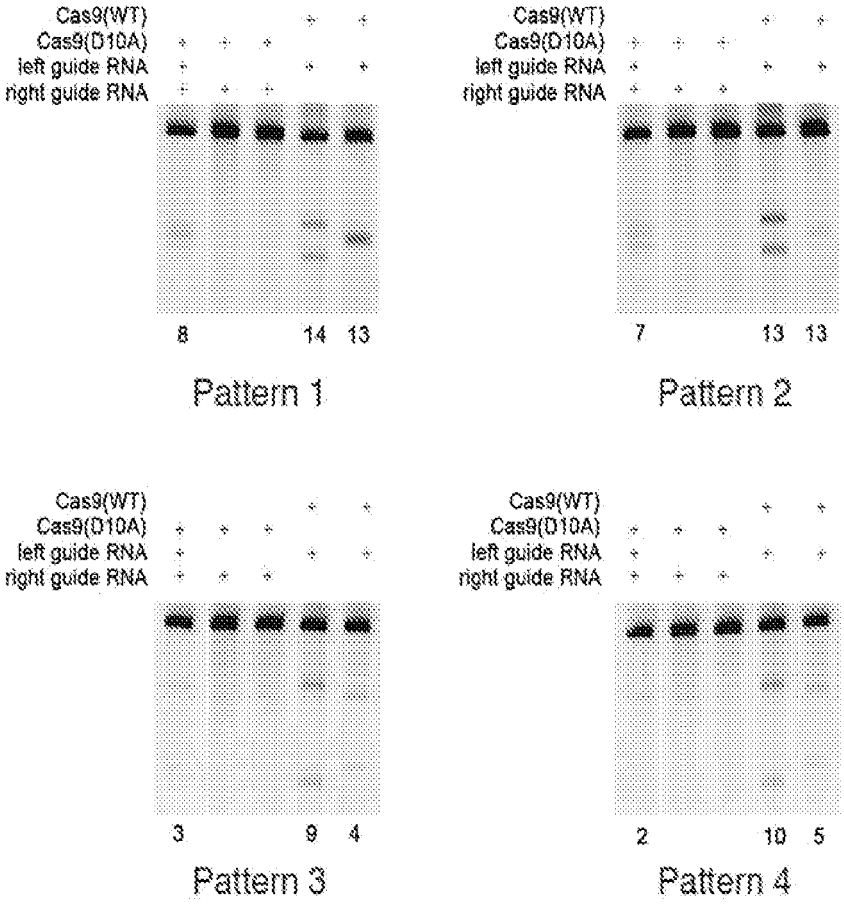


FIG. 51

Reverse PCR primers for generating U6-guide RNA expression Cassette (pair with U6 forward primer)

FZ9260	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 1
FZ9261	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 2
FZ9262	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 3
FZ9263	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 4
FZ9264	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTGGCGCATTGCCACGAAGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 5
FZ9265	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTCCGAGCAGAAGAAGAAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 6
FZ9266	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTCCGAGCAGAAGAAGAAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 7
FZ9267	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 8
FZ9268	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGCAGAAGAAGAAGGGCTCCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 9
FZ9269	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACATCAACCGGTGGCGCAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 10
FZ9270	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGGCCCAGGTGAAGGTGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 11
FZ9271	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCCTCCCTGGCCCAGGTGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 12
FZ9272	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 13
FZ9273	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 14
FZ9274	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGTGAAGGTGTGGTTCCAGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 15
FZ9275	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGACGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 16
FZ9276	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGGCCGAGGTGAAGGTGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 17
FZ9277	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 18
FZ9278	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 19
FZ9279	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGAGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 20
FZ9280	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACCTTCACCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 21
FZ9281	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACACCCTAGTCATTGGAGGTGAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 22
FZ9282	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCAGAGCAGCCACTGGGGCCTGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 23
FZ9283	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACCTTCACCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 24
FZ9284	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCCATTGGCCTGCTTCGTGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 1
FZ9285	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATTGGCCTGCTTCGTGGCAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 2
FZ9286	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCTCCTCCAGCTTCTGCCGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 3
FZ9287	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCTCCAGCTTCTGGCCGTTTGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 4
FZ9288	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATTGGCCTGCTTCGTGGCAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 5
FZ9289	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCAGCAAGCAGCACTCTGCCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 6
FZ9290	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTTCTTCTTCTGCTCGGACTCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 7
FZ9291	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACACCGGAGGACAAAGTACAAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 8
FZ9292	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCTTCTTCTGCTCGGACTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 9
FZ9293	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTGATGTGATGGGAGCCCTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 10
FZ9294	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGCCAGGGAGGGAGGGGCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 11
FZ9295	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGACGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 12
FZ9296	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCGGTTCTGGAACCACACCTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 13
FZ9297	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCAGGGAGGGAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 14
FZ9298	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCAGGGAGGGAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 15
FZ9299	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTGGAACCACACCTTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 16
FZ9300	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGAGGGAGGGGCACAGATGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 17
FZ9301	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGCCAGGGAGGGAGGGGCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 18
FZ9302	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTGGAACCACACCTTCAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 19
FZ9303	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTCCAGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 20
FZ9304	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGACAAAGTACAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 21
FZ9305	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCAAACCCACGAGGGCAGAGTGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 22
FZ9306	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTTTCTCATCTGTGCCCCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 23
FZ9307	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAACGGCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 24

FIG. 52

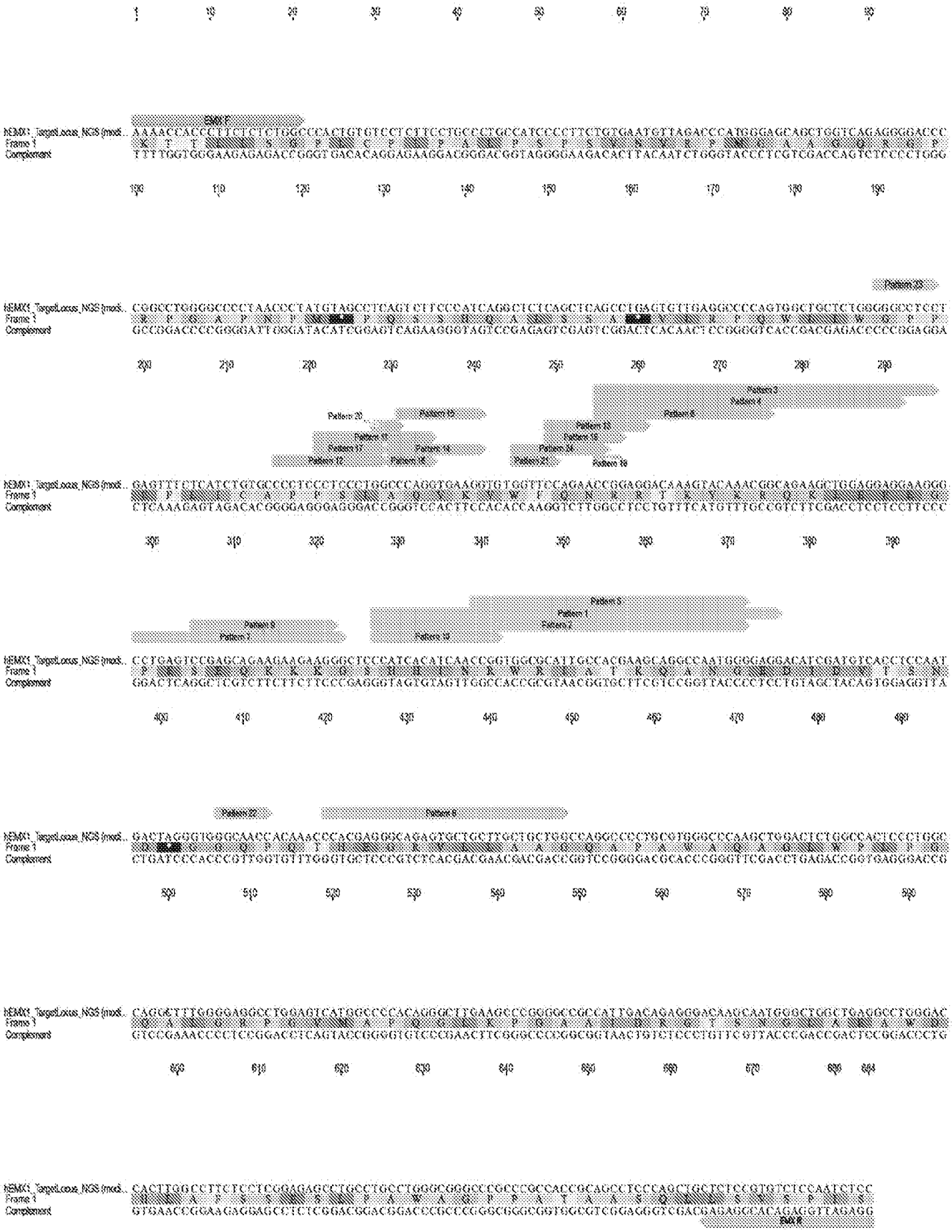
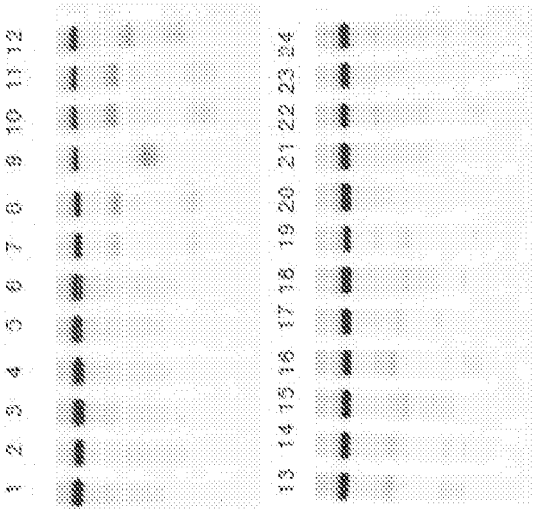


FIG. 53



lane #	Cas9 to use	Left gRNA	Right gRNA	Overhang (D10A) - number of bases protruding from 5'
1	D10A	left 23	left 12	-36
2	D10A	right 4	left 9	-25
3	D10A	left 23	right 23	-16
4	D10A	right 7	left 10	-15
5	D10A	right 16	left 3	-8
6	D10A	right 22	right 6	26
7	D10A	left 12	right 16	31
8	D10A	left 12	right 13	34
9	D10A	left 10	right 1	38
10	D10A	right 23	right 16	51
11	D10A	right 23	right 13	54
12	D10A	left 3	right 7	57
13	D10A	left 12	right 4	65
14	D10A	left 12	right 3	69
15	D10A	left 3	right 10	76
16	D10A	right 23	right 4	85
17	D10A	left 12	right 9	95
18	D10A	left 12	right 10	115
19	D10A	right 23	right 10	135
20	D10A	left 12	right 2	145
21	D10A	left 12	left 22	181
22	D10A	right 23	left 22	201
23	D10A	left 12	right 6	222
24	D10A	right 23	right 6	242

FIG. 54

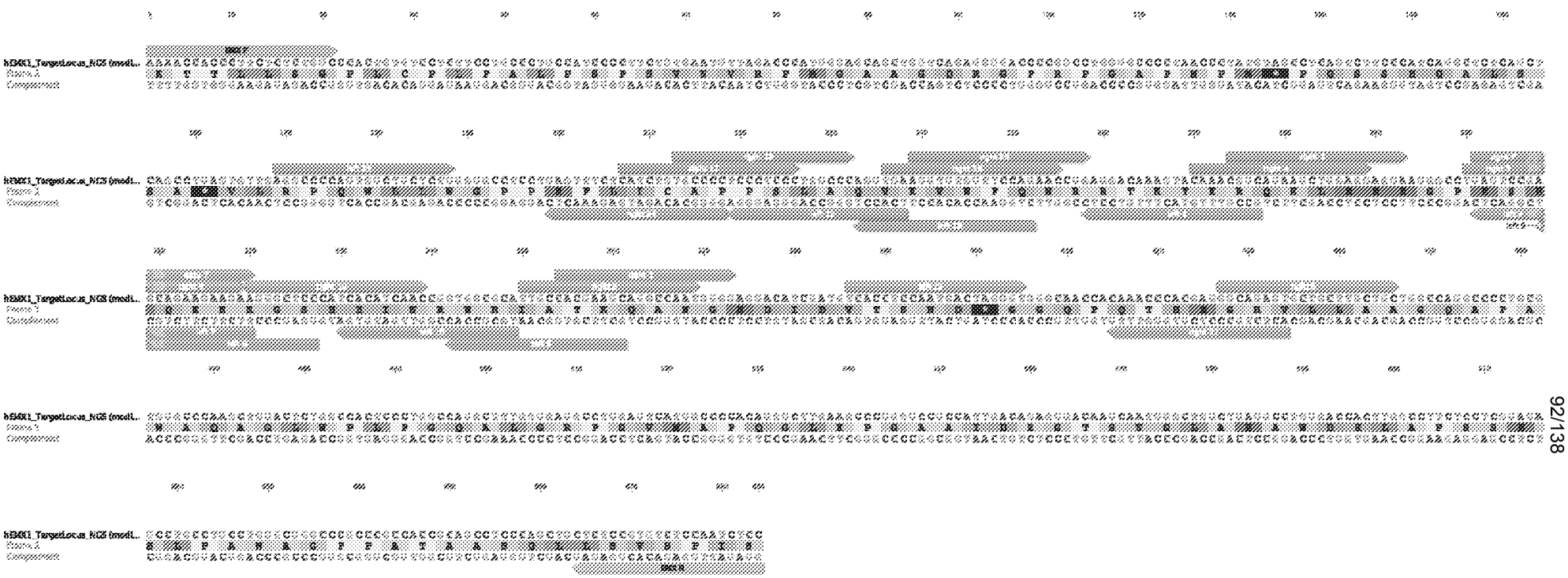


FIG. 55

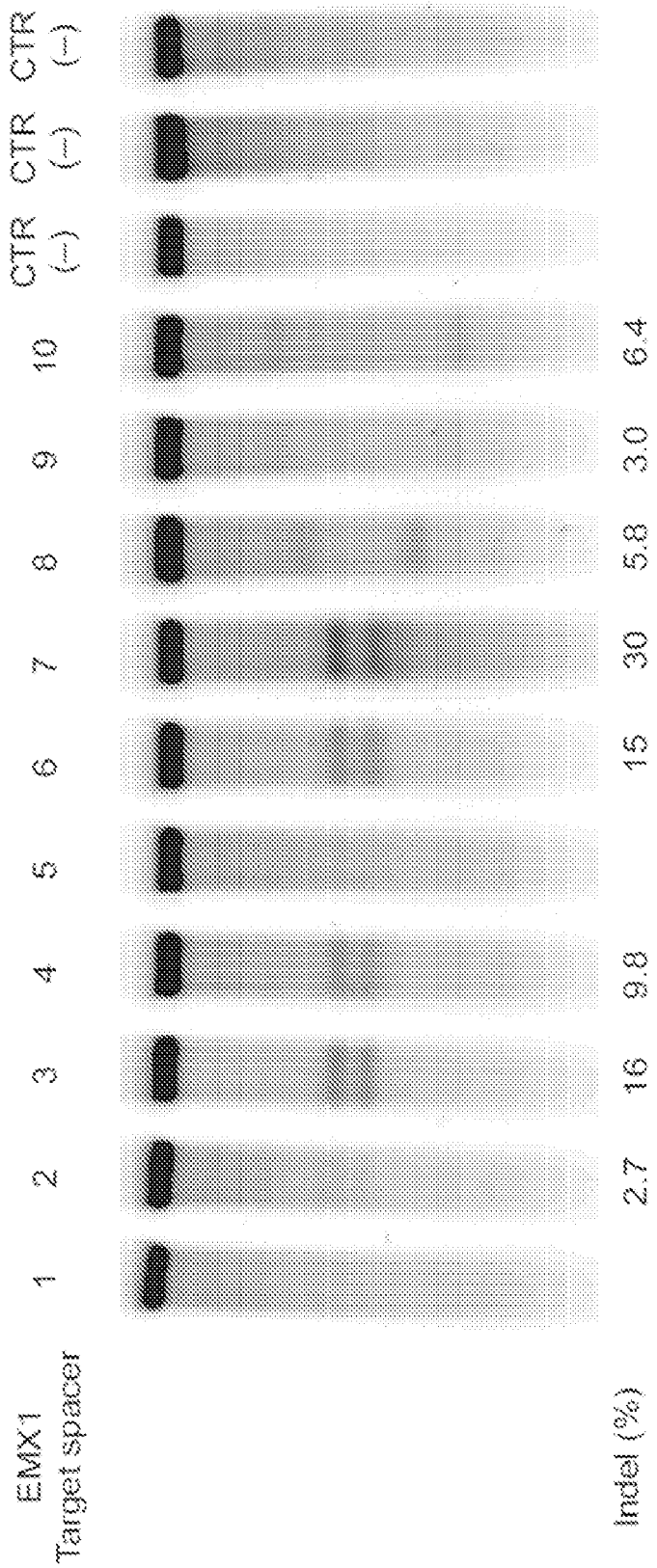

EMX1
Target spacer
1
2
3
4
5
6
7
8
9
10
CTR (–)
CTR (–)
CTR (–)
Indel (%)
2.7
16
9.8
15
30
5.8
3.0
6.4

FIG. 56

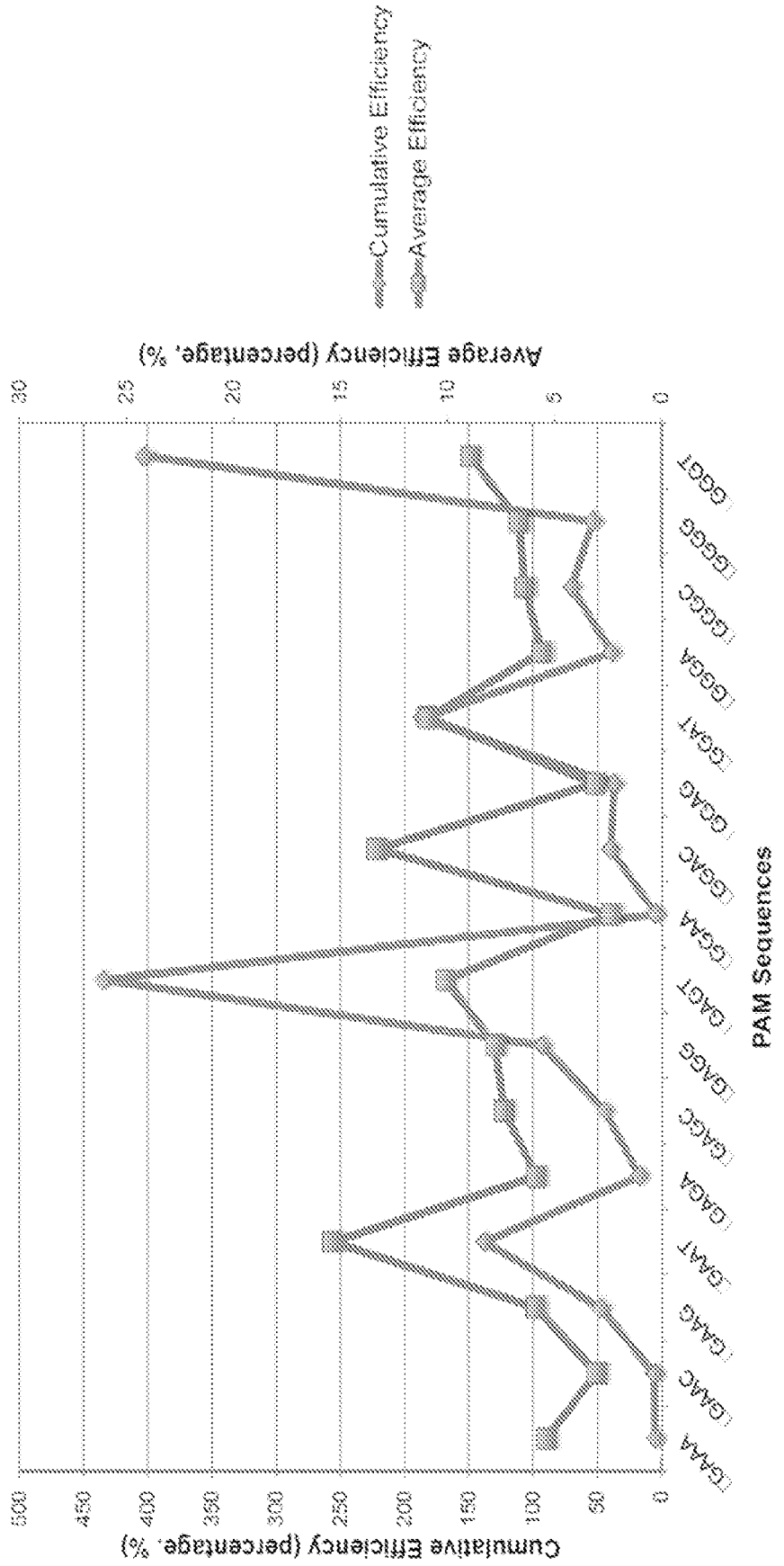

Cumulative Efficiency
Average Efficiency
Average Efficiency (percentage, %)
Cumulative Efficiency (percentage, %)
PAM Sequences
GAAA
GAAC
GAAG
GAAT
GACA
GACC
GACG
GACT
GAGA
GAGC
GAGG
GAGT
GATA
GATC
GATG
GATT
GGAA
GGAC
GGAG
GGAT
GGCA
GGCC
GGCG
GGCT
GGGA
GGGC
GGGG
GGGT

FIG. 57

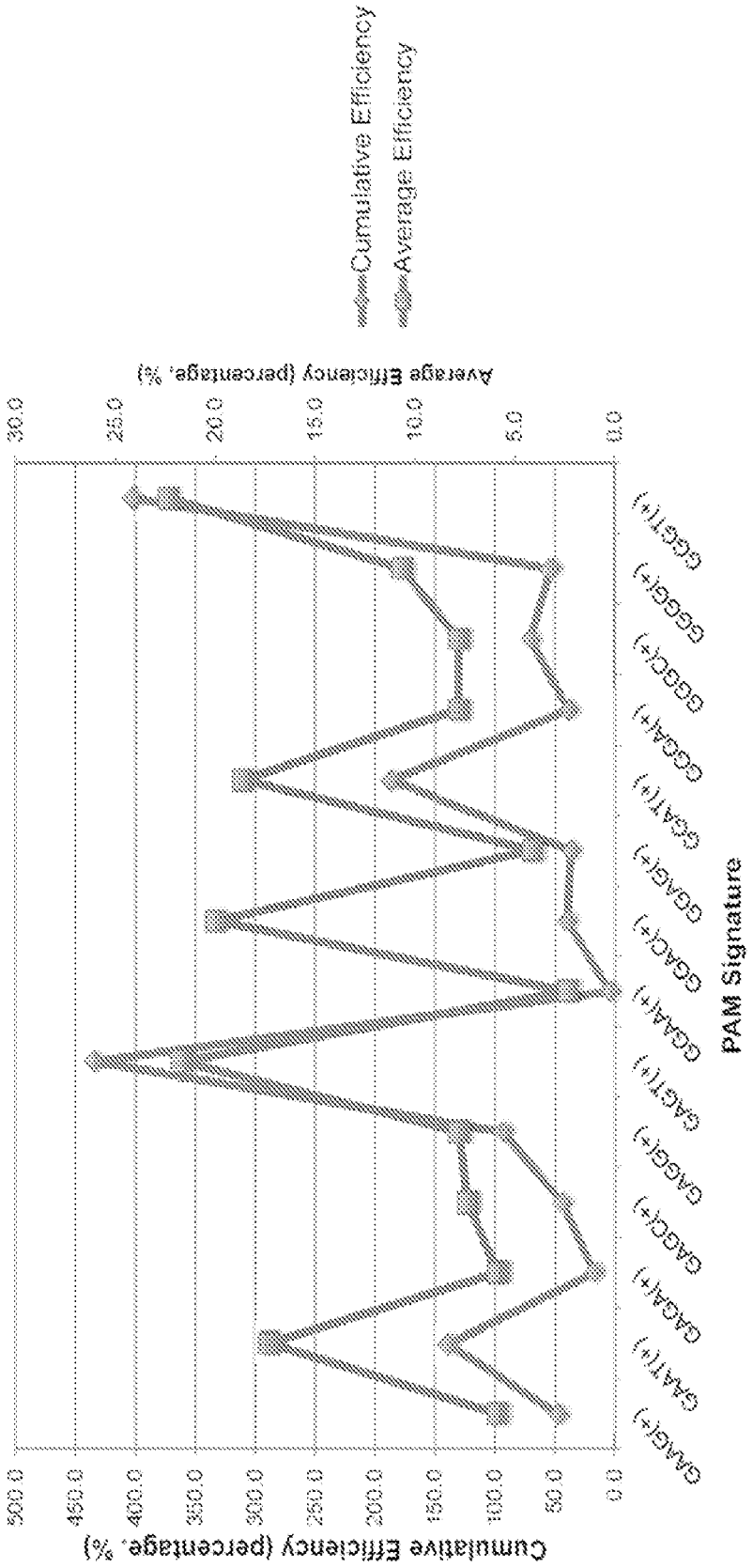

Cumulative Efficiency
Average Efficiency
Cumulative Efficiency (percentage, %)
Average Efficiency (percentage, %)
PAM Signature
GAAGA(+)
GAATA(+)
GAGAA(+)
GAGCA(+)
GAGGA(+)
GACTA(+)
GCAAA(+)
GCACA(+)
GCAGA(+)
GCATA(+)
GGAAA(+)
GGGCA(+)
GGGGA(+)
GGGTA(+)

FIG. 58

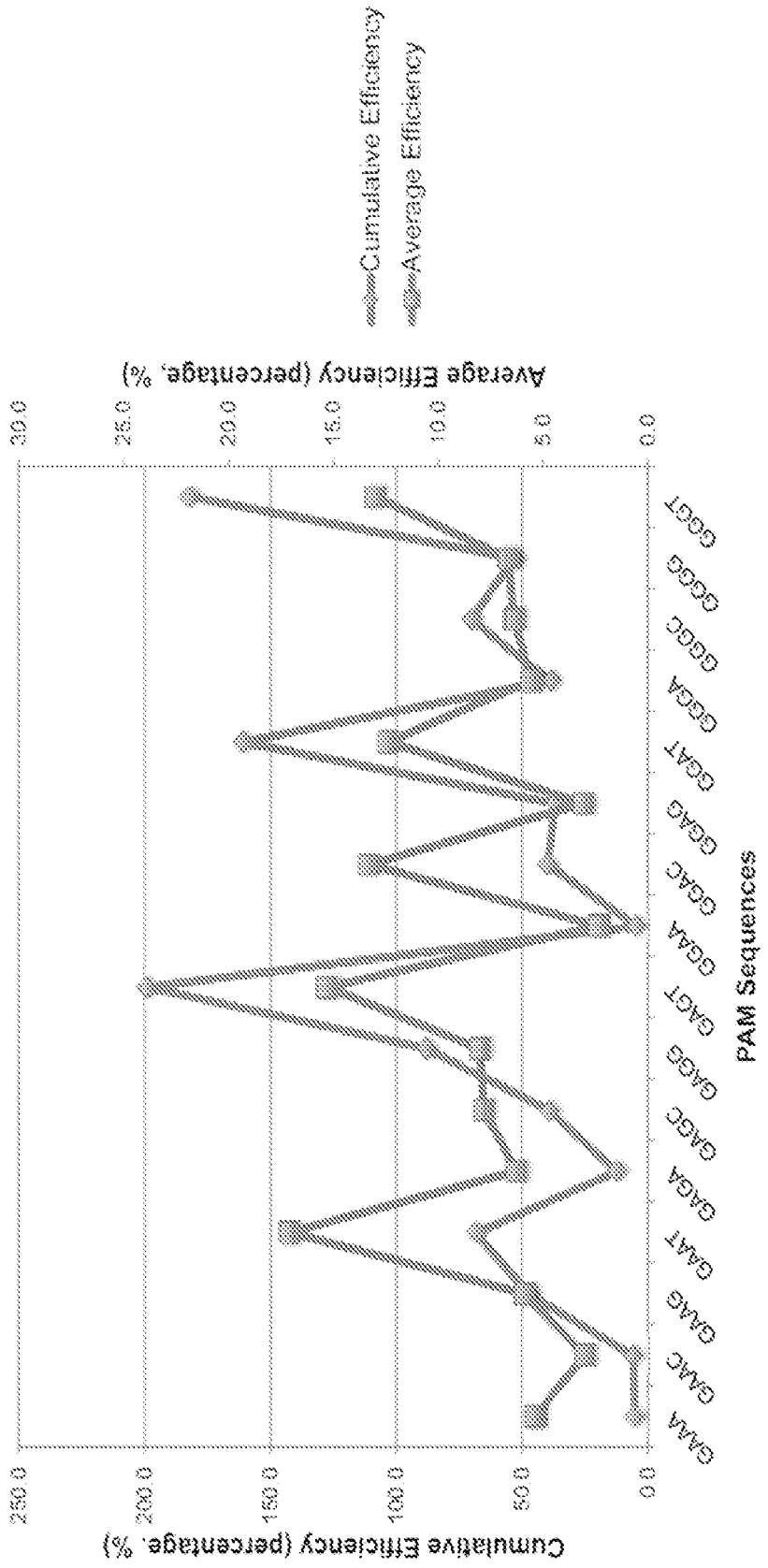

Cumulative Efficiency
Average Efficiency
Average Efficiency (percentage, %)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Cumulative Efficiency (percentage, %)
250.0
200.0
150.0
100.0
50.0
0.0
GAAA
GAAC
GAAG
GAAT
GACA
GACC
GACG
GACT
GAGA
GAGC
GAGG
GAGT
GATA
GATC
GATG
GATT
GCAA
GCAC
GCAG
GCAT
GCCA
GCCC
GCCG
GCCT
PAM Sequences

FIG. 59

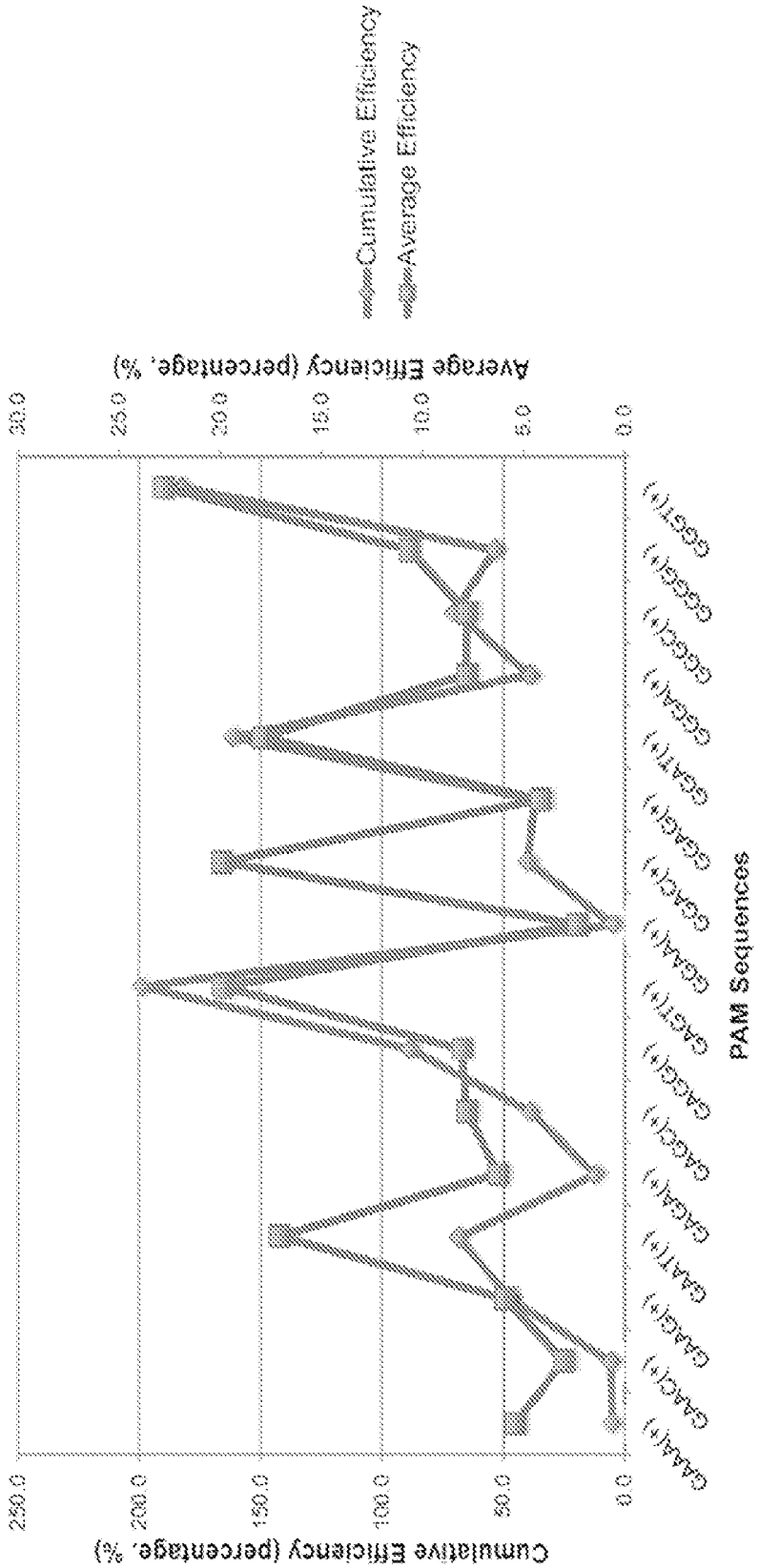

Cumulative Efficiency
Average Efficiency
Average Efficiency (percentage, %)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Cumulative Efficiency (percentage, %)
250.0
200.0
150.0
100.0
50.0
0.0
PAM Sequences
GAAAN
GAACN
GAAGN
GAATN
GACAN
GACCN
GACGN
GACTN
GAGAN
GAGCN
GAGGN
GAGTN
GATAN
GATCN
GATGN
GATTN
GCAAN
GCACN
GCAGN
GCATN
GCGAN
GCGCN
GCGGN
GCGTN

FIG. 60

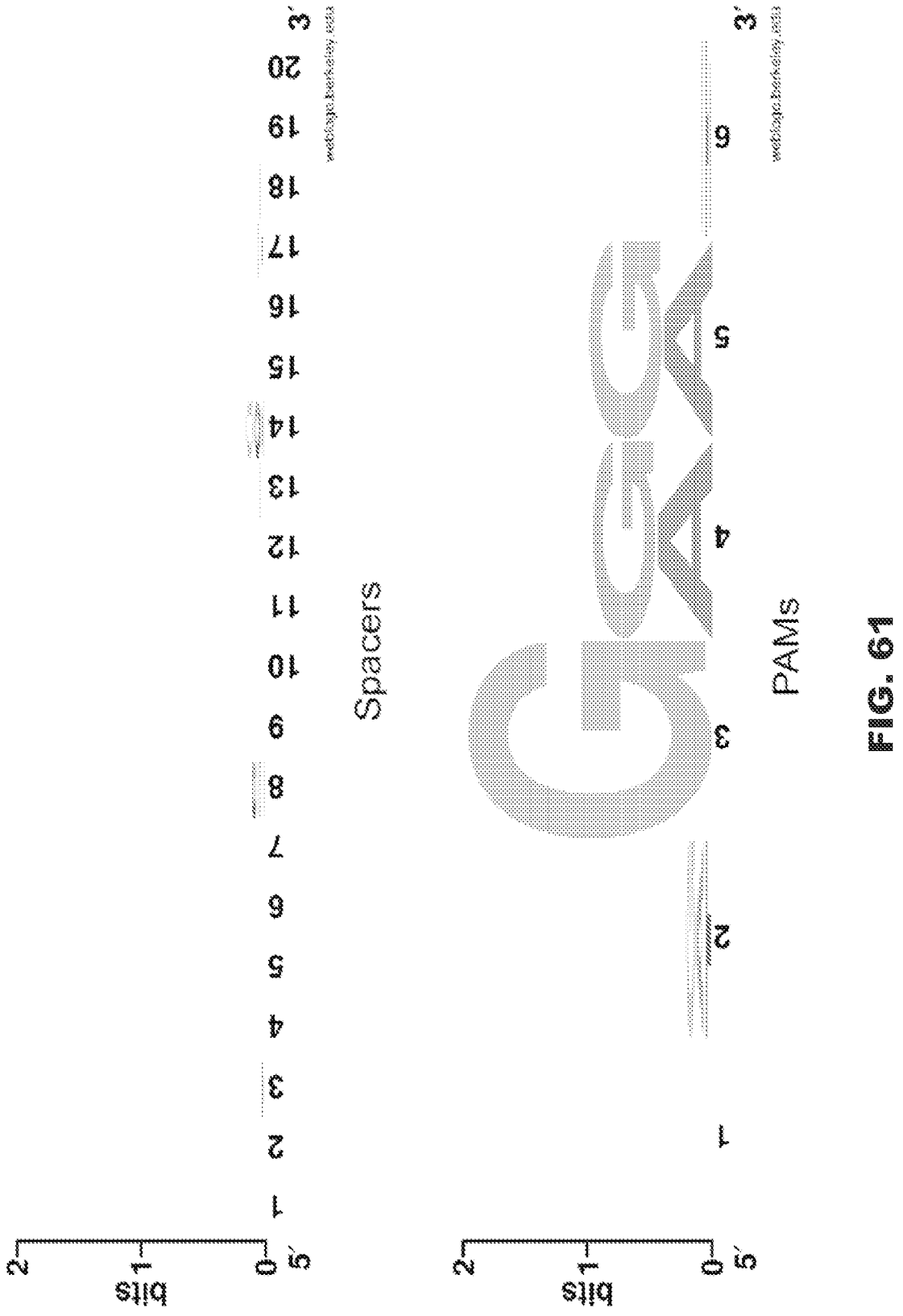


FIG. 61

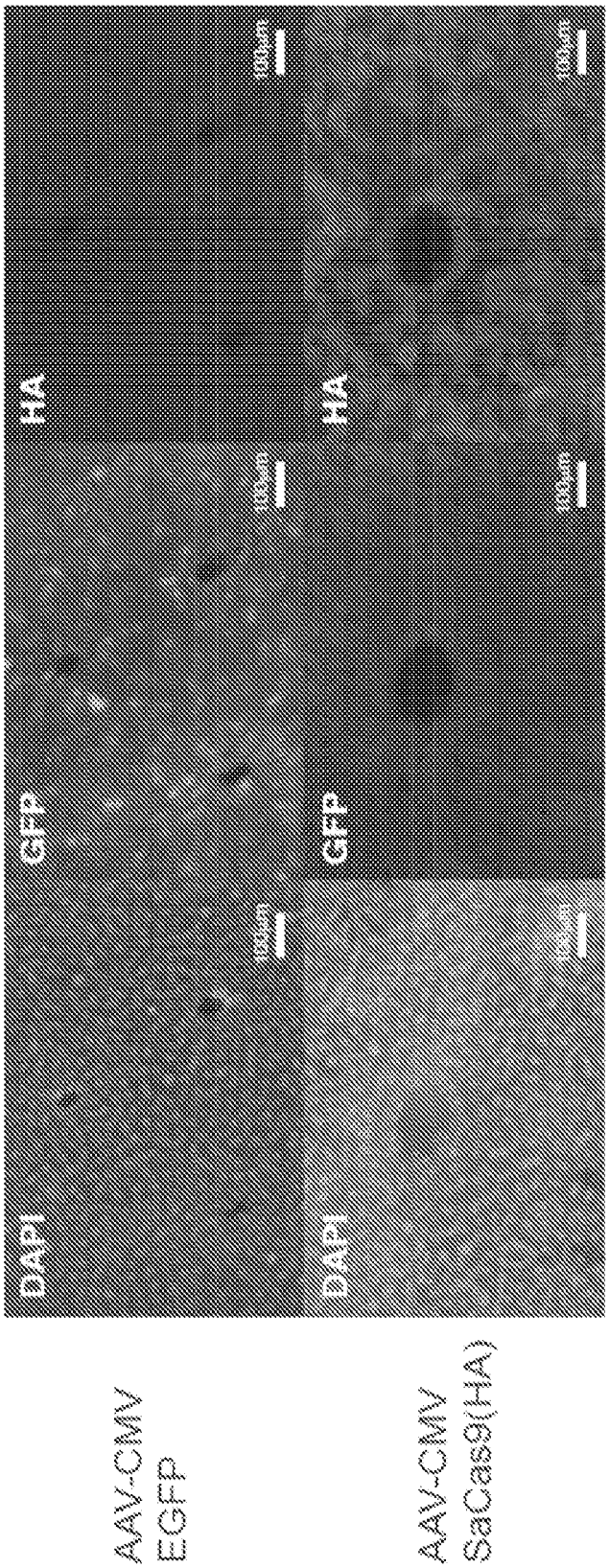


FIG. 62

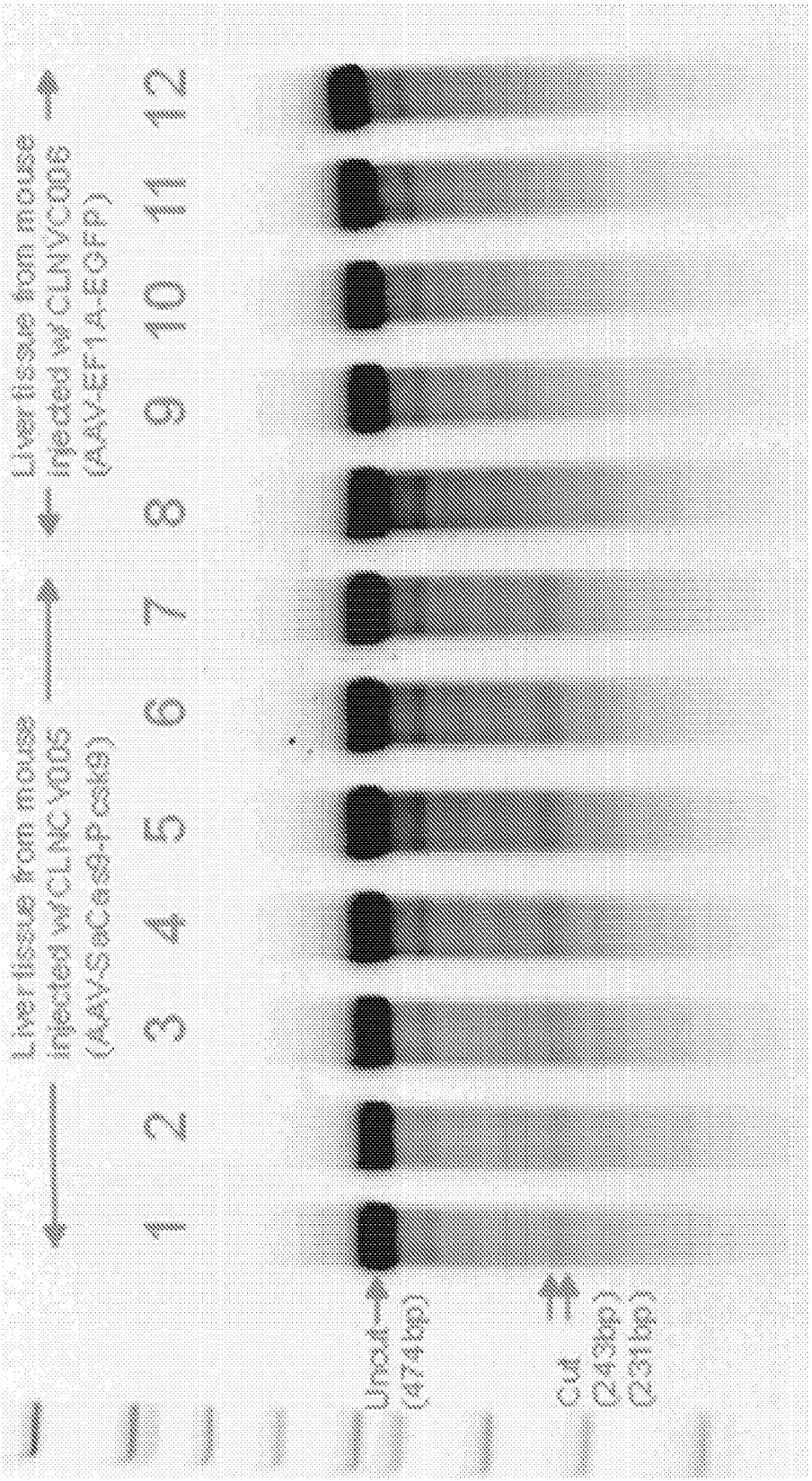

Liver tissue from mouse injected w/ CLNC-Y005 (AAVS-sCas9-P-cas9)
Liver tissue from mouse injected w/ CLNVC006 (AAV-EF1A-EGFP)
1 2 3 4 5 6 7 8 9 10 11 12
Undigested (474bp)
Cut (243bp) (231bp)

FIG. 63

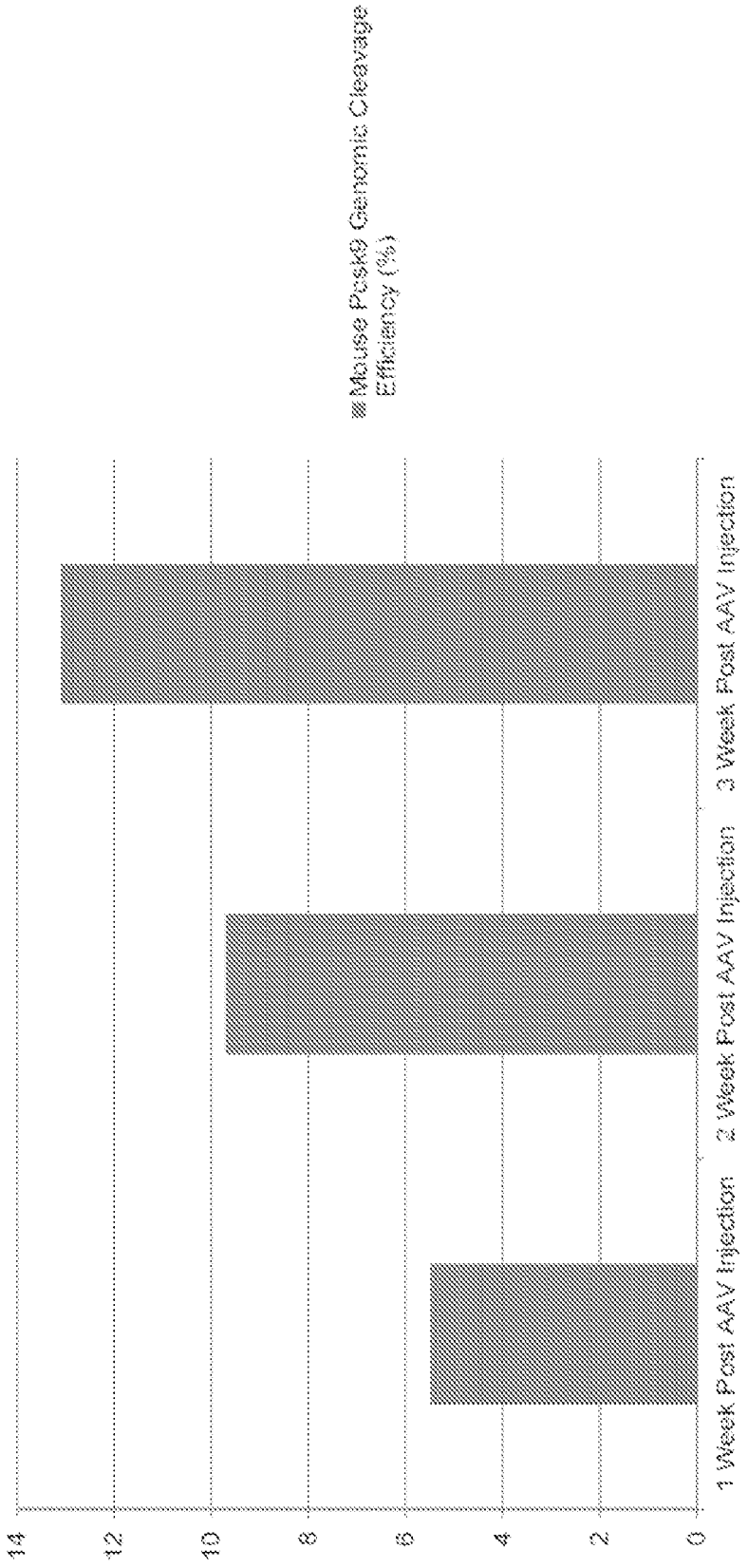

Mouse Pcsk9 Genomic Cleavage Efficiency (%)
14
12
10
8
6
4
2
0
1 Week Post AAV Injection
2 Week Post AAV Injection
3 Week Post AAV Injection

FIG. 64

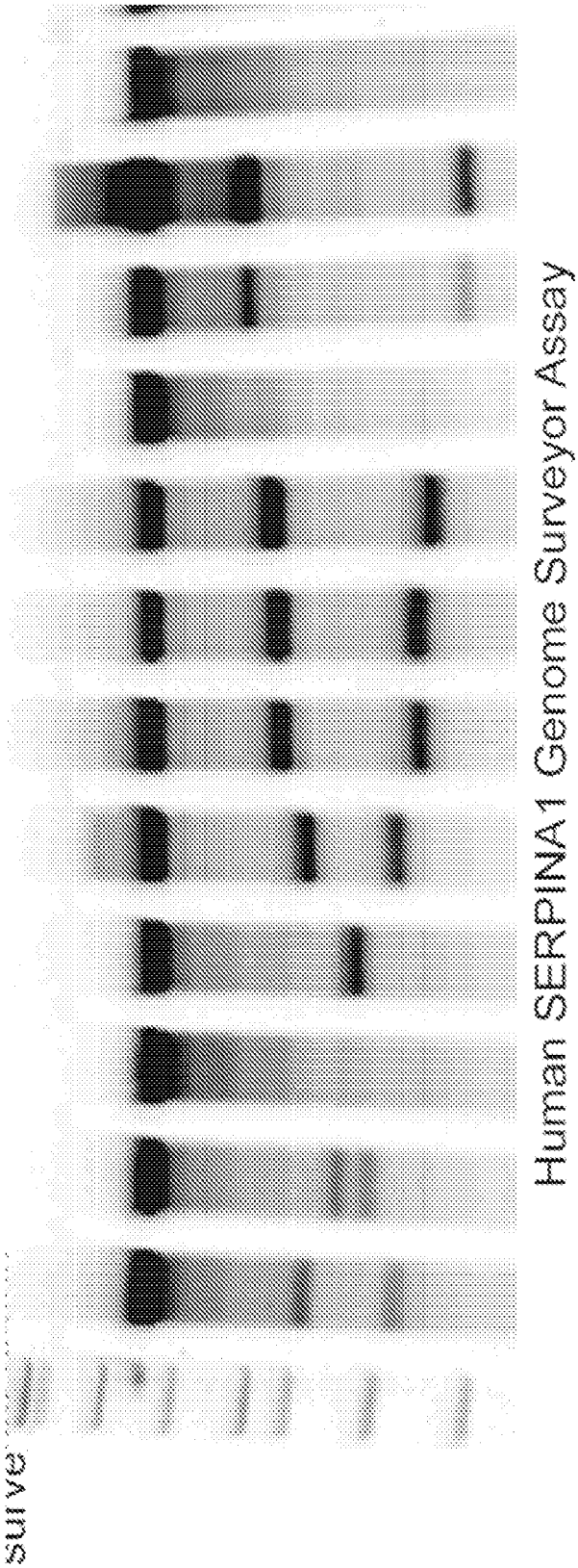


FIG. 65

Genome Target Sapcers	Genome Cleavage Efficiency (%)
MmHmgcr-Spacer 1	15.4
MmHmgcr-Spacer 2	18.3
MmHmgcr-Spacer 3	6.1
MmHmgcr-Spacer 4	6.5
MmHmgcr-Spacer 5	11.8
MmHmgcr-Spacer 6	5.8

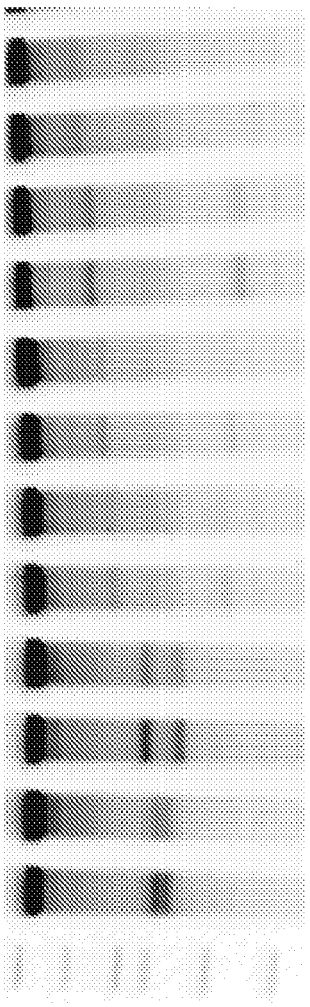
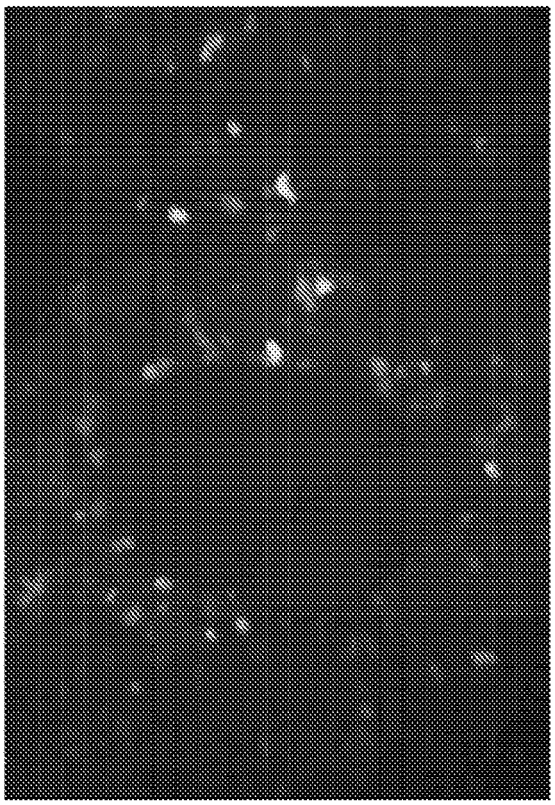


FIG. 66



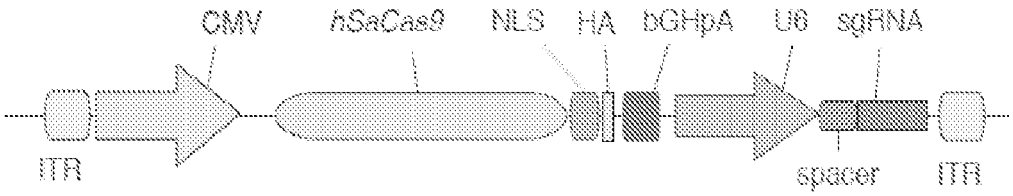
TBG Version



CMV version

FIG. 67

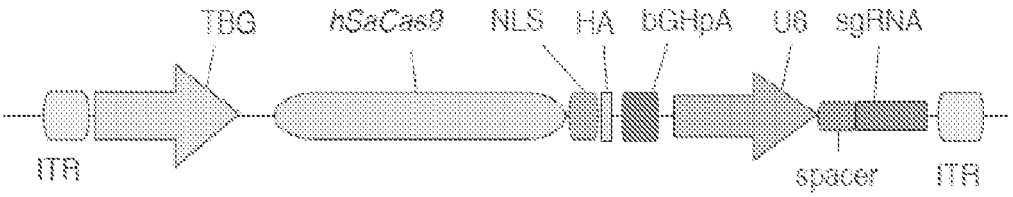
A



pAAV-CMV-SaCas9-U6-sgRNA architecture

(CMV: ubiquitous cytomegalovirus promoter)

B

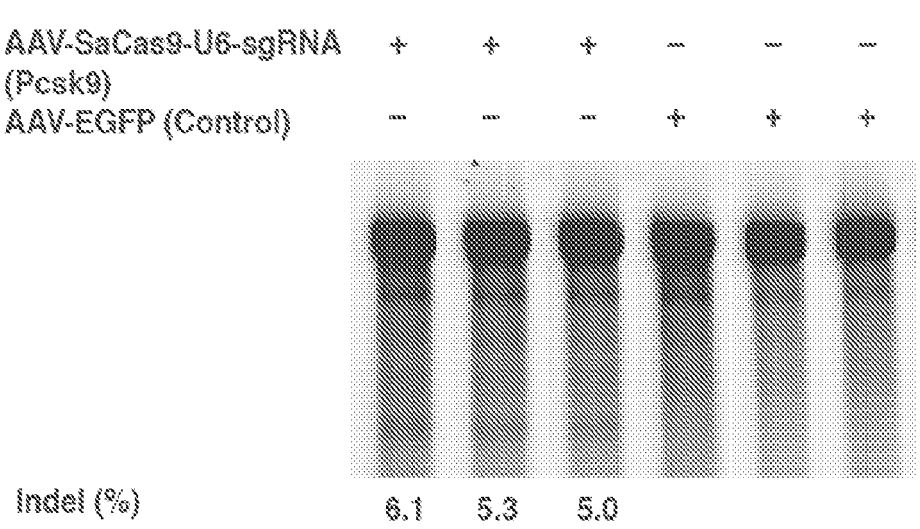


pAAV-TBG-SaCas9-U6-sgRNA architecture

(TBG: Thyroxine-binding globulin liver-specific promoter)

FIG. 68

A


AAV-SaCas9-U6-sgRNA (Pcsk9)
AAV-EGFP (Control)
+ + + – – –
– – – + + +
Indel (%)
6.1 5.3 5.0

B

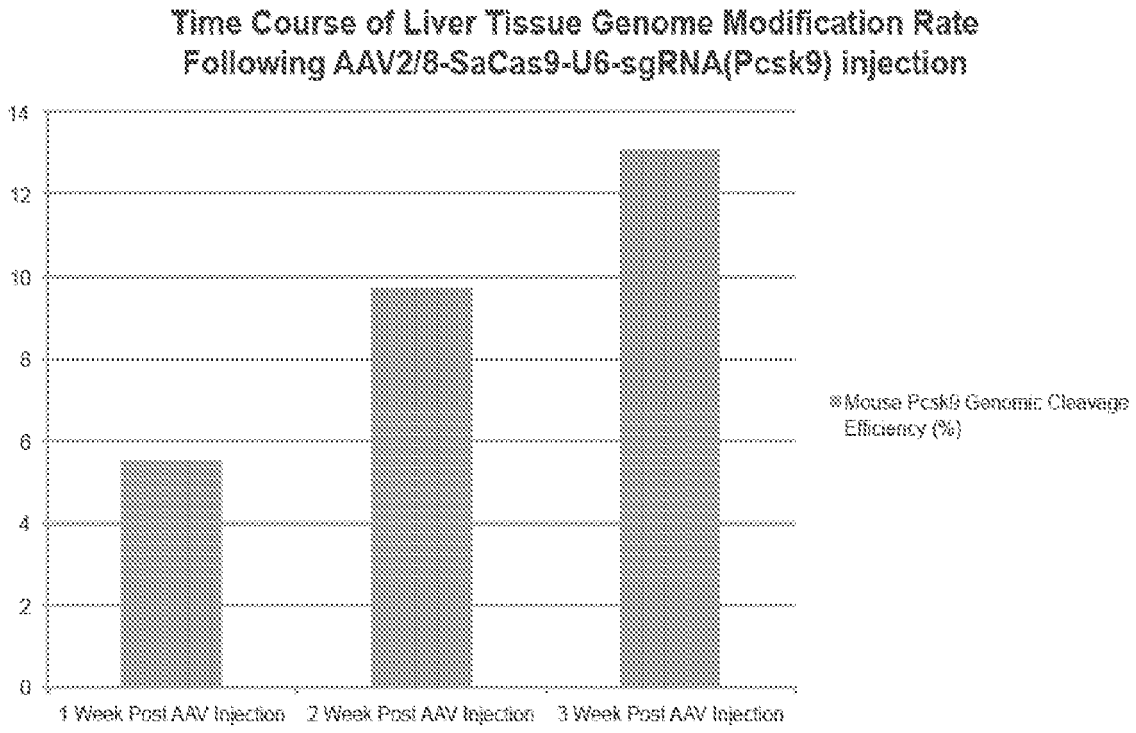

Time Course of Liver Tissue Genome Modification Rate
Following AAV2/8-SaCas9-U6-sgRNA(Pcsk9) injection
14
12
10
8
6
4
2
0
1 Week Post AAV Injection
2 Week Post AAV Injection
3 Week Post AAV Injection
※Mouse Pcsk9 Genomic Cleavage Efficiency (%)

FIG. 69

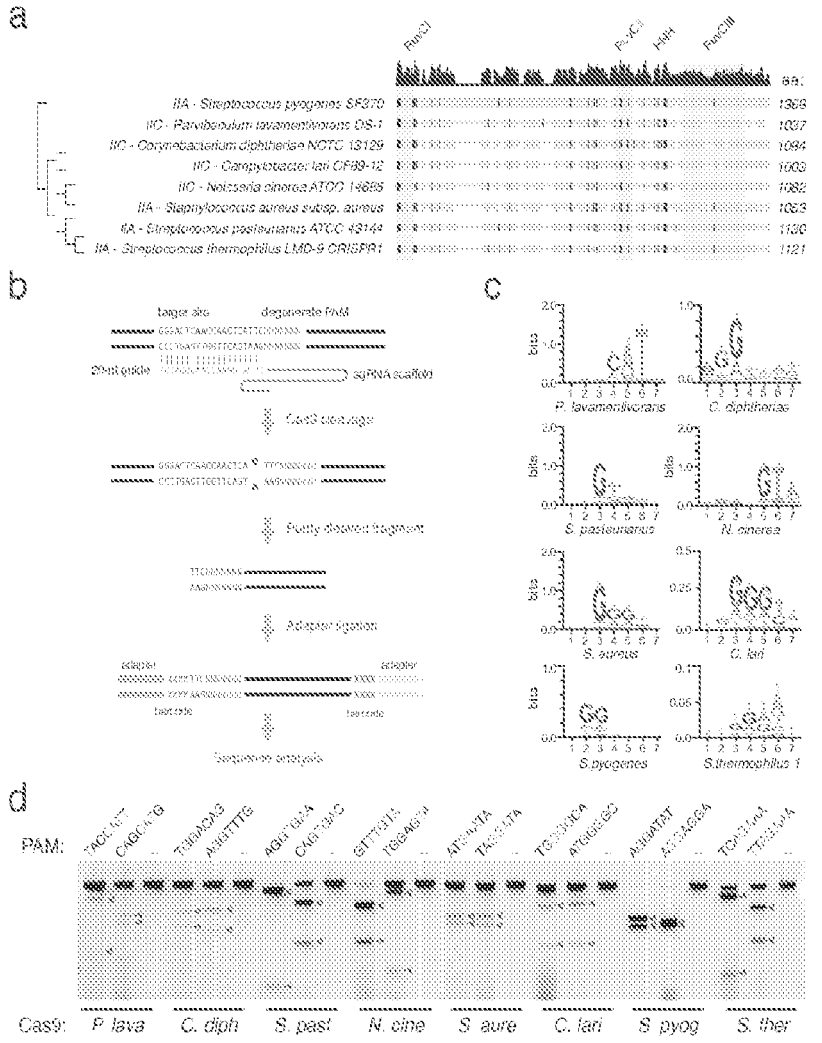

a
FunCI
FunCII
HdH
FunCIII
IIA - Streptococcus pyogenes SF370
IIC - Parvibaculum lavamentivorans DS-1
IIC - Corynebacterium diphtheriae NCTC 13129
IIC - Olsenella uli DSM 7084
IIC - Neisseria cinerea ATCC 14685
IIA - Staphylococcus aureus subsp. aureus
IIA - Streptococcus pasteurianus ATCC 43144
IIA - Streptococcus thermophilus LMD-9 CRISPR1
b
target site
degenerate PAM
crRNA guide
sgRNA scaffold
Cas9 cleavage
Partly digested fragment
Adapter ligation
adapter
barcode
Sequencing adapters
c
P. lavamentivorans
C. diphtheriae
S. pasteurianus
N. cinerea
S. aureus
C. lari
S.pyogenes
S.thermophilus 1
bits
d
PAM:
Cas9:
P. lava
C. diph
S. past
N. cine
S. aure
C. lari
S. pyog
S. ther

FIG. 70A-D

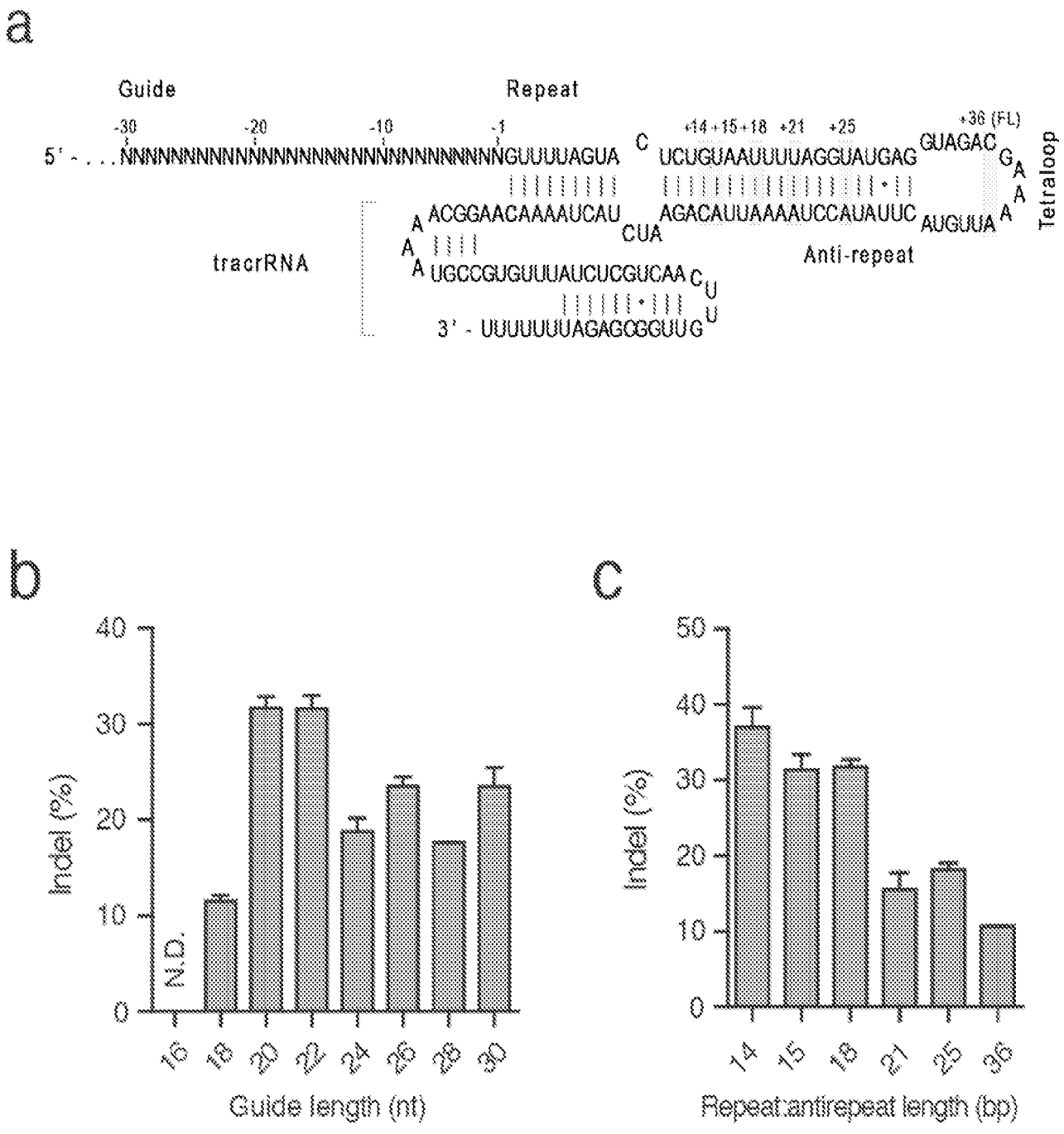

a
Guide
Repeat
-30
-20
-10
-1
+14 +15 +18
+21
+25
+36 (FL)
5' - . . . NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGUUUUAGUA C UCUGUAAUUUUAGGUAUGAG GUAGAC G A A
A ACGGAACAAAAUCAU CUA AGACAUUAAAAUCCAUAUUC AUGUUA A
tracrRNA
A UGCCGUGUUUAUCUCGUCAA C U
Anti-repeat
Tetraloop
3' - UUUUUUUAGAGCGGUUG U
b
Indel (%)
N.D.
Guide length (nt)
c
Indel (%)
Repeat:antirepeat length (bp)

FIG. 71A-C

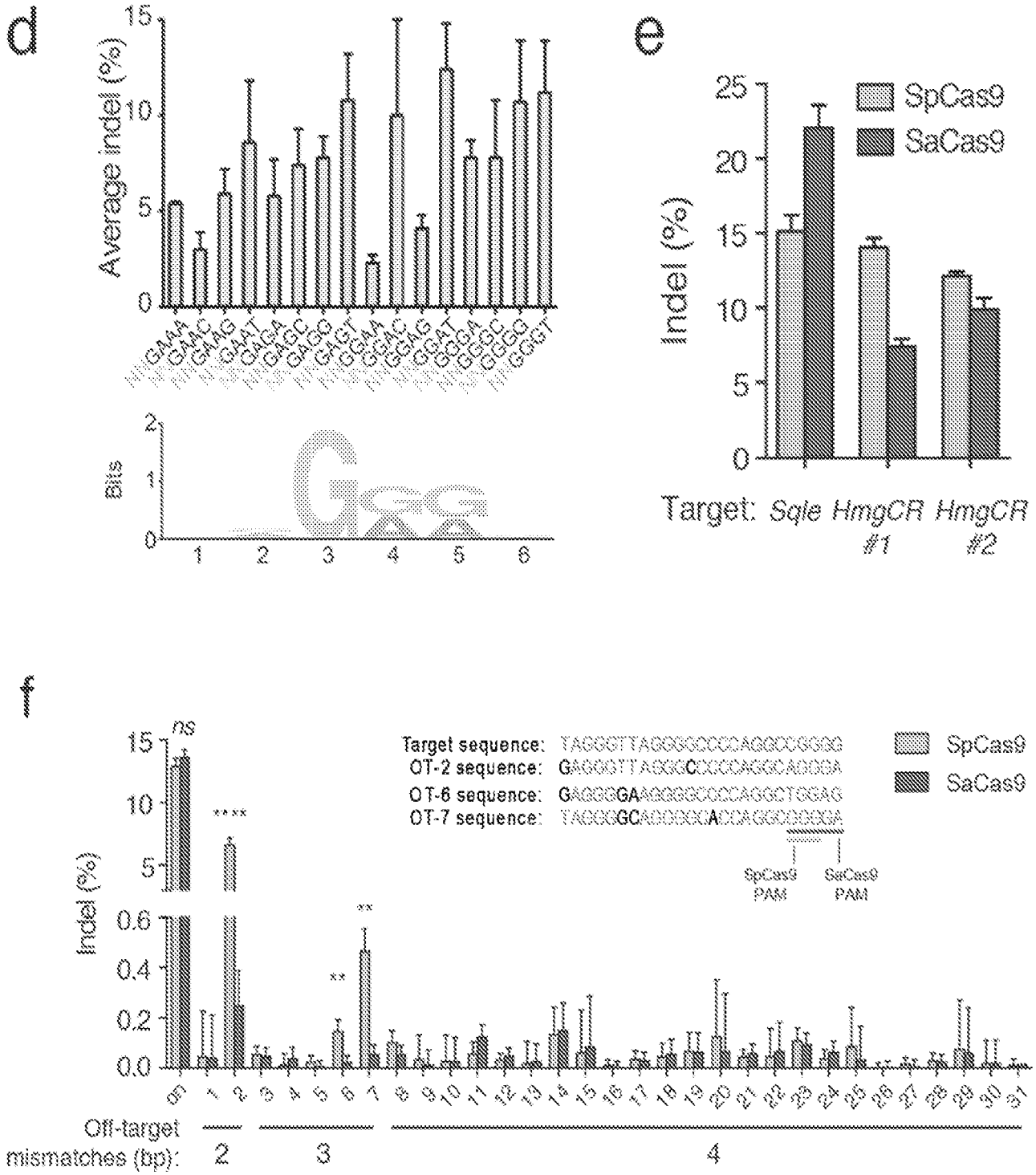
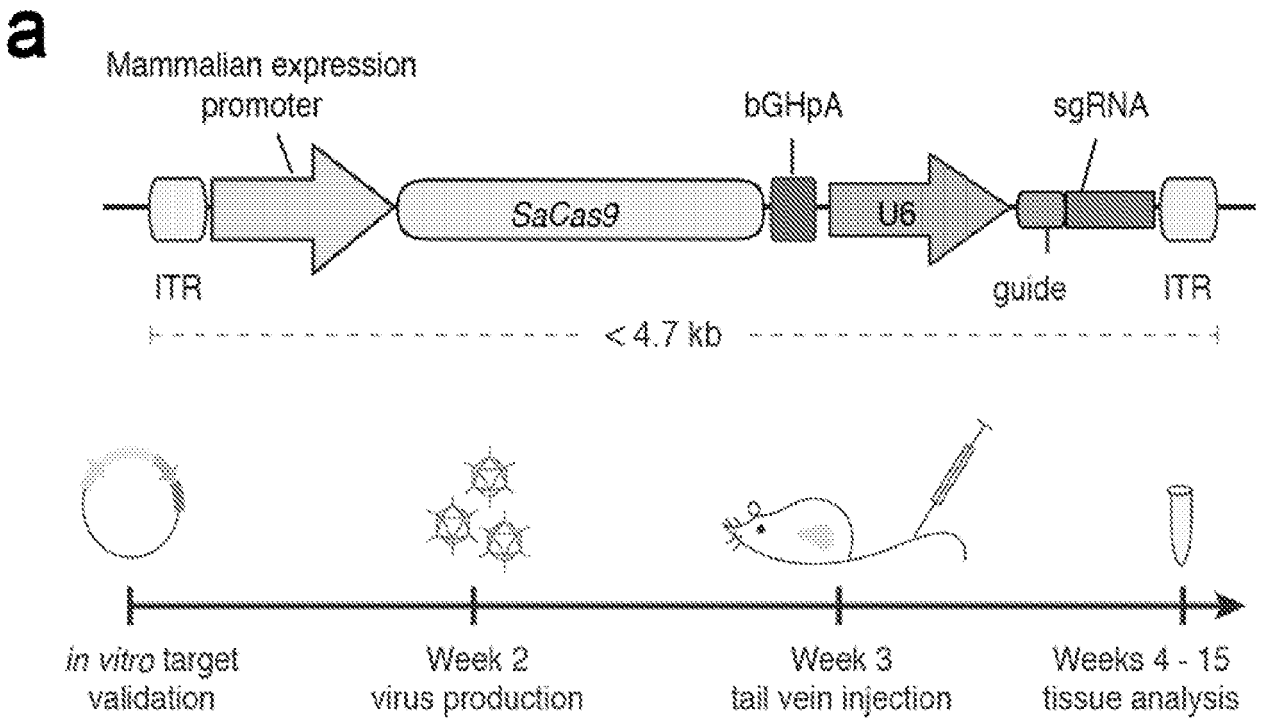

d
Average indel (%)
15
10
5
0
GAAA
GAAC
GAAG
GAAT
GAGA
GAGC
GAGG
GAGT
GGAA
GGAC
GGAG
GGAT
GGGA
GGGC
GGGG
GGGT
Bits
2
1
0
1 2 3 4 5 6
e
Indel (%)
25
20
15
10
5
0
SpCas9
SaCas9
Target: Sqle HmgCR #1 HmgCR #2
f
Indel (%)
ns
****
**
**
15
10
5
0.6
0.4
0.2
0.0
Target sequence: TAGGGTTAGGGGCCCCAGGCCGGGG
OT-2 sequence: GAGGGTTAGGGCCCCCAGGCAGGGA
OT-6 sequence: GAGGGGAAGGGGCCCCAGGCTGGAG
OT-7 sequence: TAGGGGCAGGGCCCACCAGGCCGGGA
SpCas9 PAM
SaCas9 PAM
SpCas9
SaCas9
Off-target mismatches (bp):
2
3
4

FIG. 71D-F


a
Mammalian expression promoter
bGHpA
sgRNA
SaCas9
U6
ITR
guide
ITR
< 4.7 kb
in vitro target validation
Week 2 virus production
Week 3 tail vein injection
Weeks 4 - 15 tissue analysis

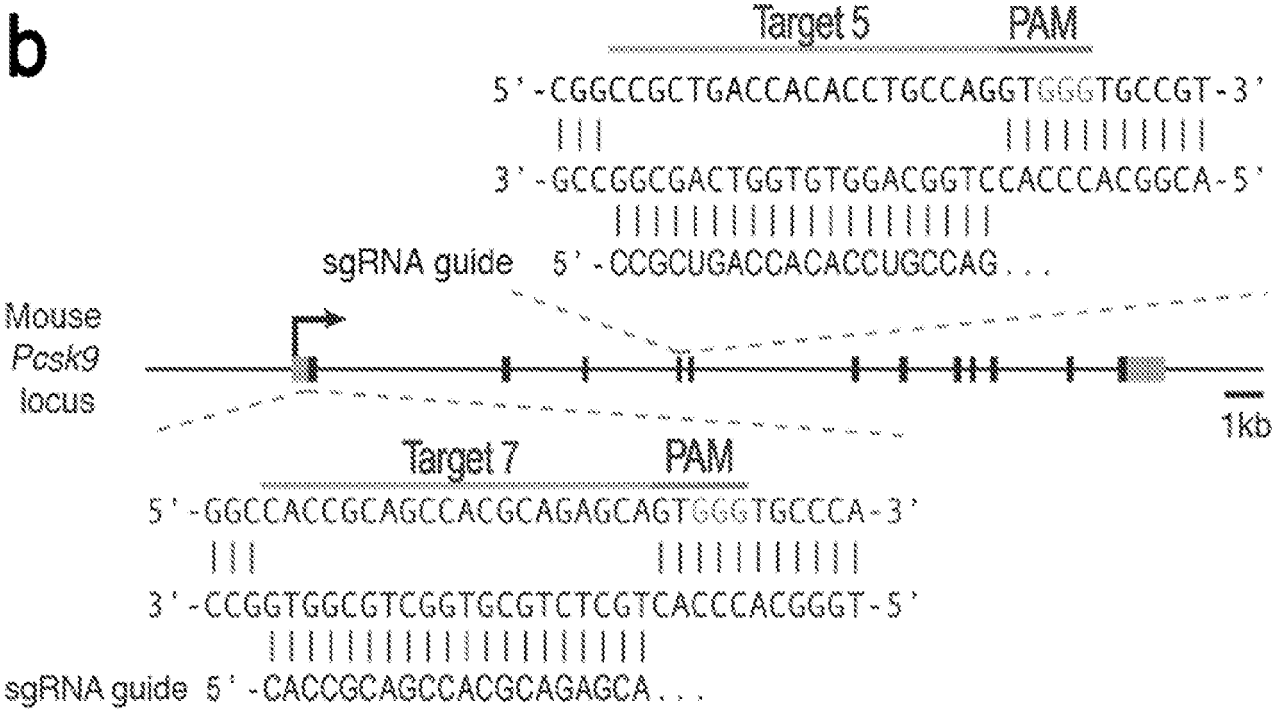

b
Target 5
PAM
5'-CGGCCGCTGACCACACCTGCCAGGTGGGTGCCGT-3'
3'-GCCGGCGACTGGTGTGGACGGTCCACCCACGGCA-5'
sgRNA guide 5'-CCGCUGACCACACCUGCCAG...
Mouse Pcsk9 locus
1kb
Target 7
PAM
5'-GGCCACCGCAGCCACGCAGAGCAGTGGGTGCCCA-3'
3'-CCGGTGGCGTCGGTGCGTCTCGTCACCCACGGGT-5'
sgRNA guide 5'-CACCGCAGCCACGCAGAGCA...

FIG. 72A-B

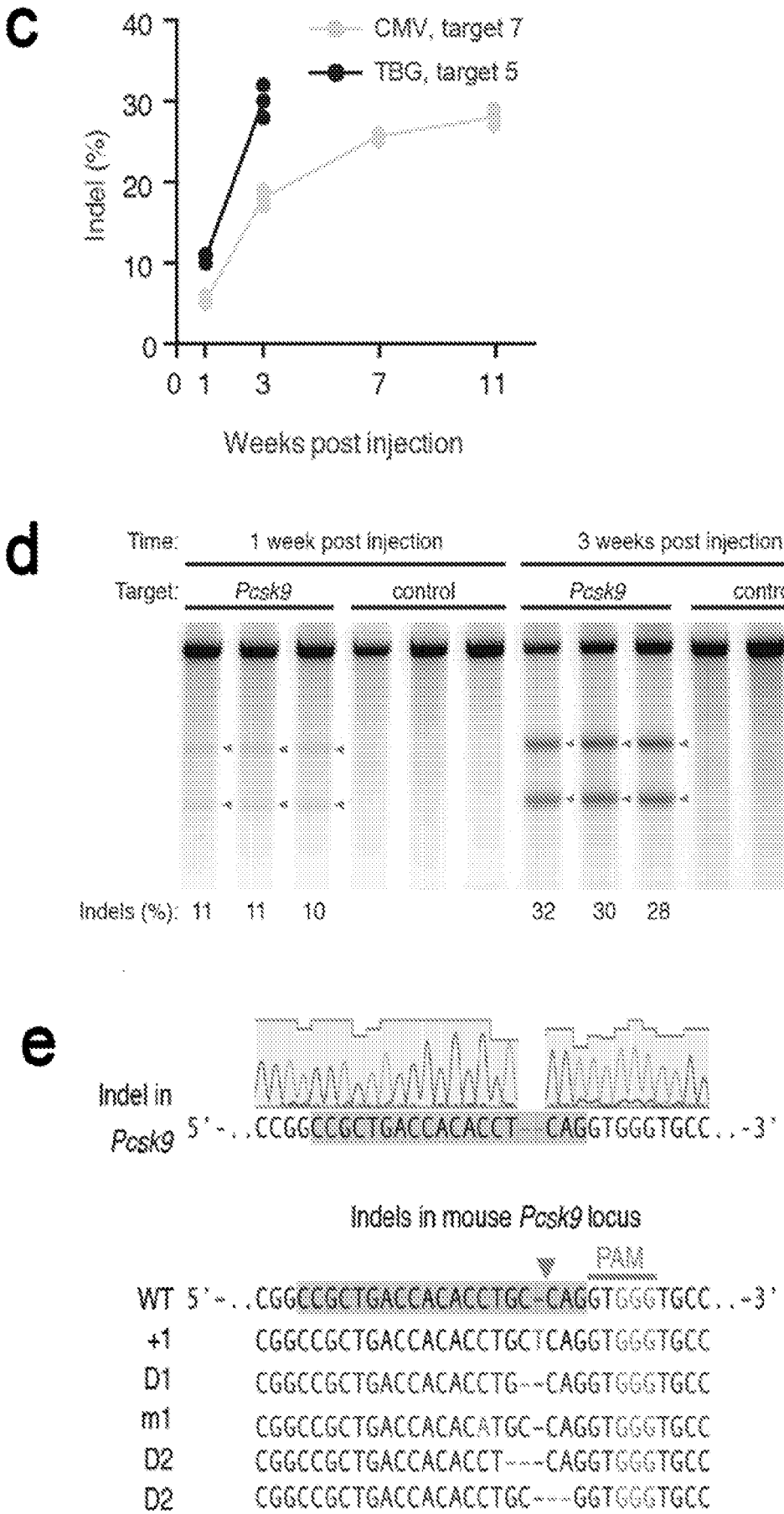

c
CMV, target 7
TBG, target 5
Indel (%)
40
30
20
10
0
0 1 3 7 11
Weeks post injection
d
Time:
1 week post injection
3 weeks post injection
Target:
Pcsk9
control
Pcsk9
control
Indels (%): 11 11 10 32 30 28
e
Indel in Pcsk9
5'-..CCGGCCGCTGACCACACCT CAGGTGGGTGCC..-3'
Indels in mouse Pcsk9 locus
PAM
WT 5'-..CGGCCGCTGACCACACCTGC-CAGGTGGGTGCC..-3'
+1 CGGCCGCTGACCACACCTGCTCAGGTGGGTGCC
D1 CGGCCGCTGACCACACCTG--CAGGTGGGTGCC
m1 CGGCCGCTGACCACACATGC-CAGGTGGGTGCC
D2 CGGCCGCTGACCACACCT---CAGGTGGGTGCC
D2 CGGCCGCTGACCACACCTGC---GGTGGGTGCC

FIG. 72C-E

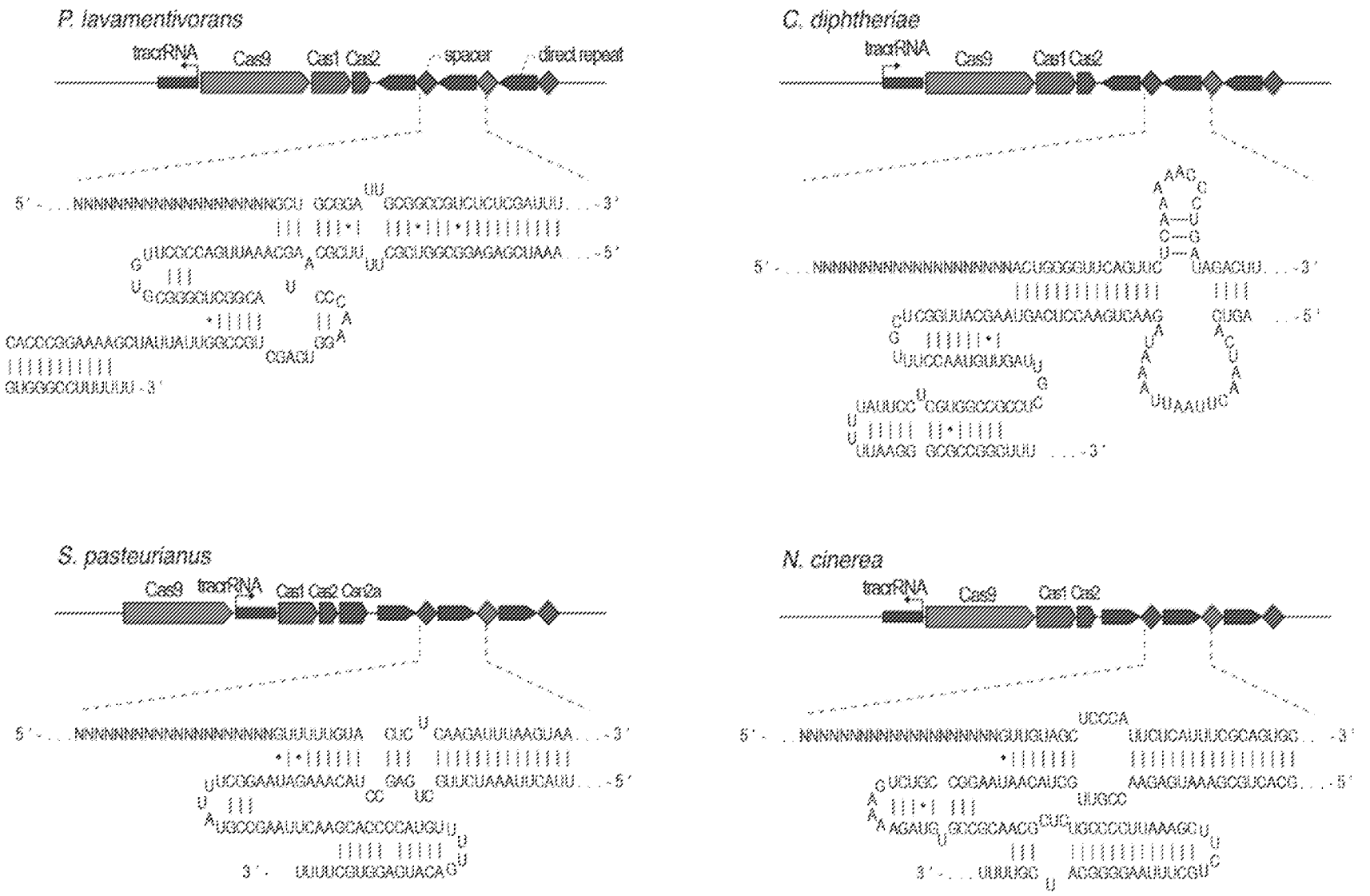

P. lavamentivorans
tracrRNA
Cas9
Cas1 Cas2
spacer
direct repeat
C. diphtheriae
tracrRNA
Cas9
Cas1 Cas2
S. pasteurianus
Cas9
tracrRNA
Cas1 Cas2 Csn2a
N. cinerea
tracrRNA
Cas9
Cas1 Cas2

FIG. 73A

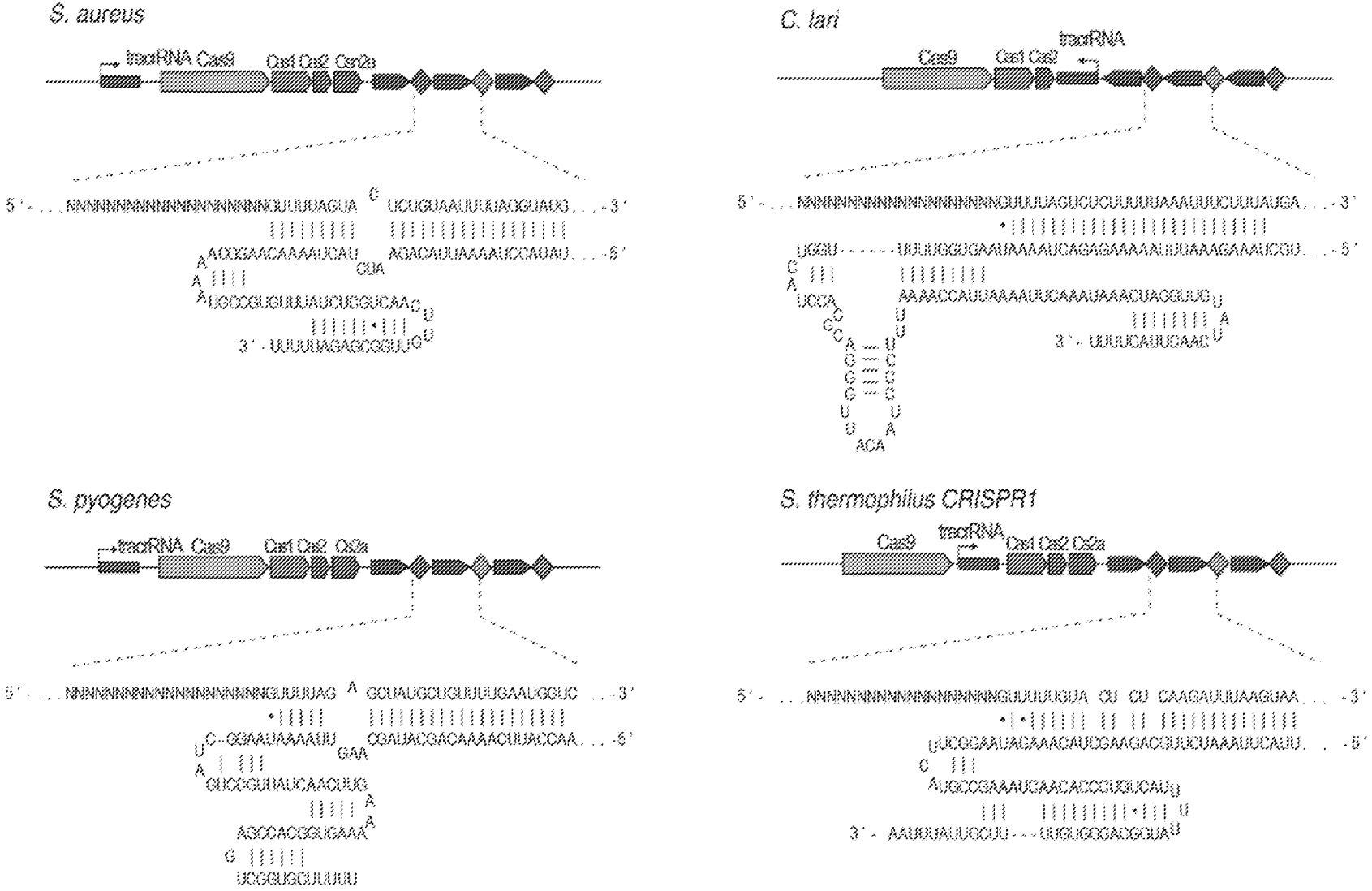

S. aureus
tracrRNA
Cas9
Csn1 Csn2
Csn2a
C. lari
Cas9
Csn1 Csn2
tracrRNA
S. pyogenes
tracrRNA
Cas9
Csn1 Csn2
Csn2a
S. thermophilus CRISPR1
Cas9
tracrRNA
Csn1 Csn2 Csn2a

FIG. 73B

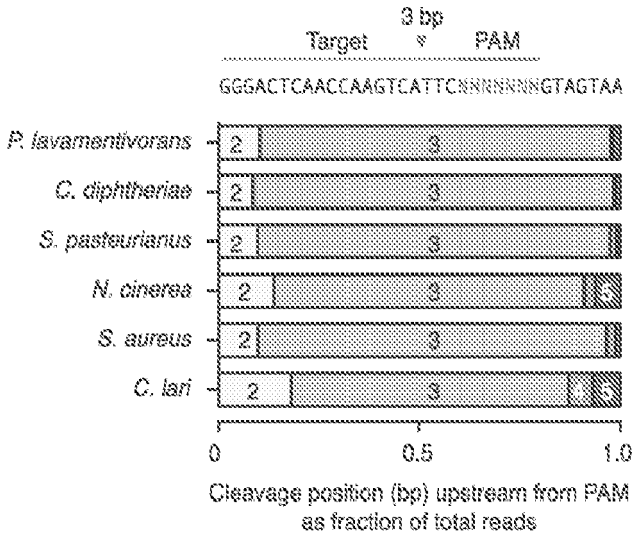

3 bp
Target
PAM
GGGACTCAACCAAGTCATTCNNNNNNNGTAGTAA
P. lavamentivorans
C. diphtheriae
S. pasteurianus
N. cinerea
S. aureus
C. lari
2
3
0
0.5
1.0
Cleavage position (bp) upstream from PAM
as fraction of total reads

FIG. 74

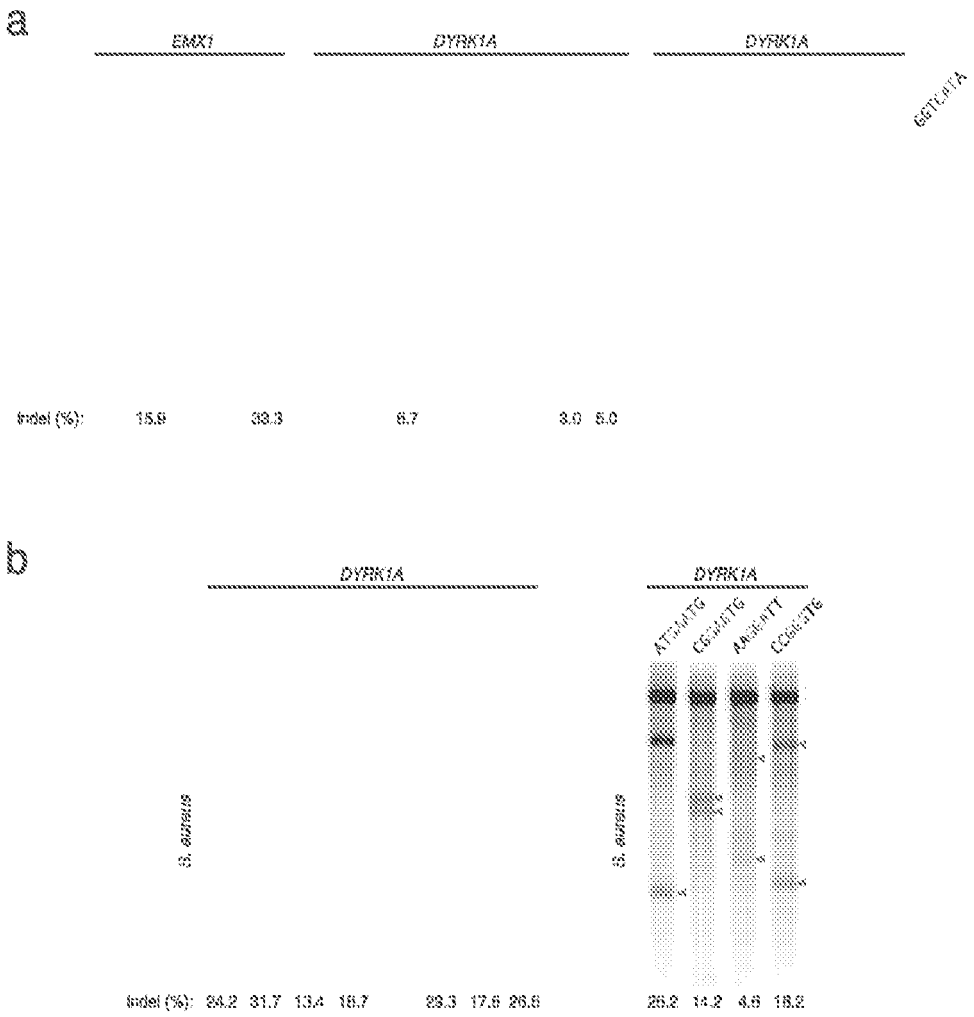
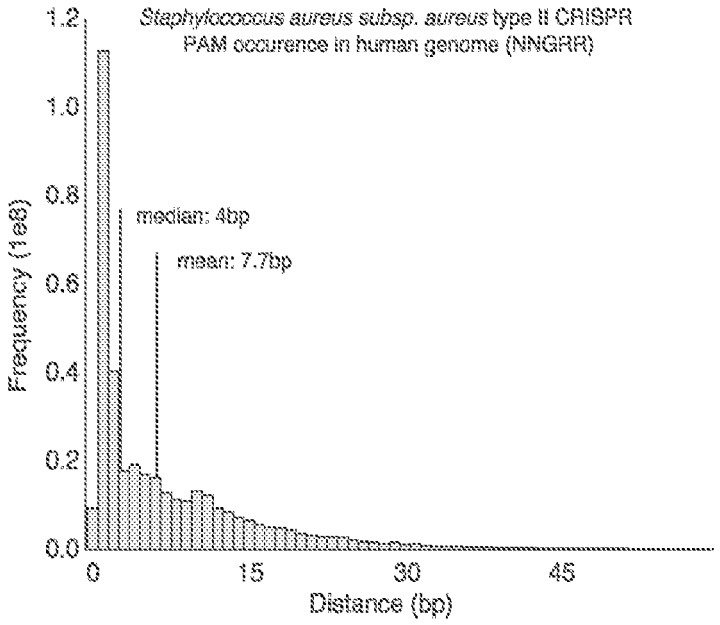

a
EMX1
DYRK1A
DYRK1A
GGTCATA
Indel (%): 15.9 38.3 6.7 3.0 6.0
b
DYRK1A
DYRK1A
ATCAATG
CGCAATG
AACAATT
CGCCATG
S. aureus
S. aureus
Indel (%): 24.2 31.7 13.4 16.7 29.3 17.6 26.6 26.2 14.2 4.6 18.2

FIG. 75A-B

a

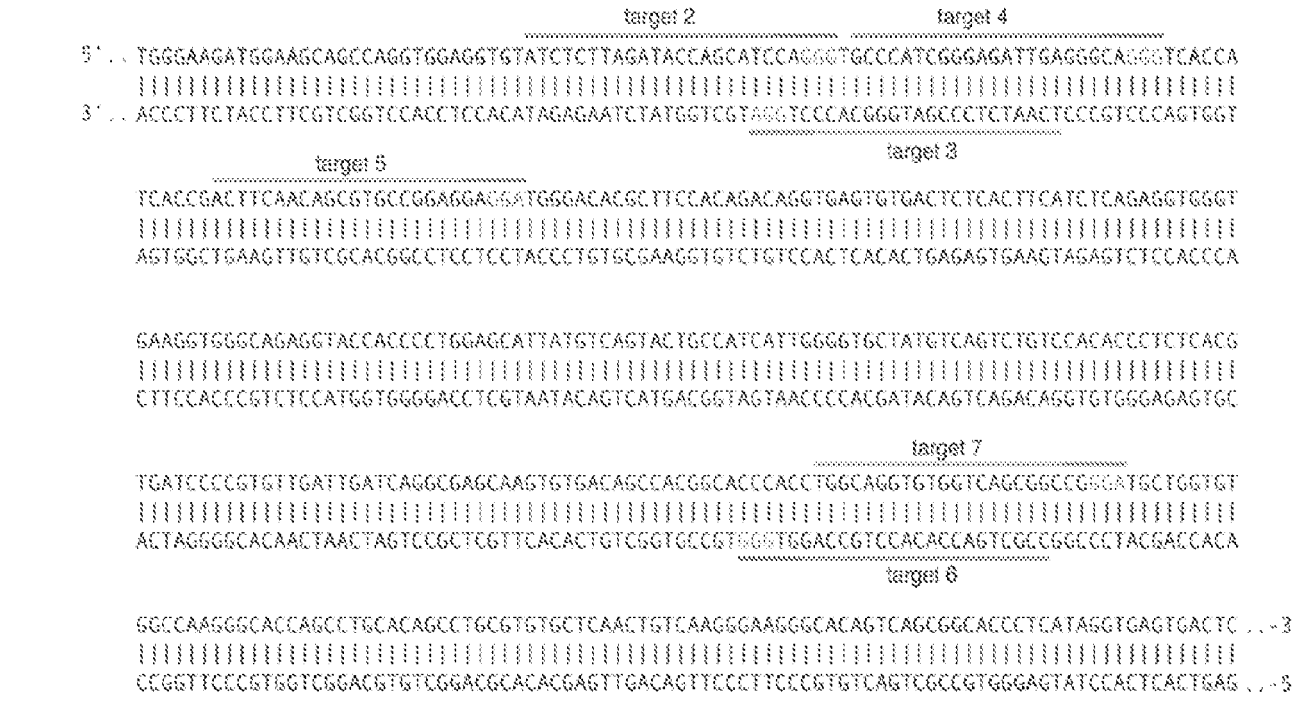


b

	NNGRR	
Chr	median	mean
1	4	7.5
2	4	7.9
3	4	7.9
4	4	8.3
5	4	8.0
6	4	7.9
7	4	7.7
8	4	7.8
9	4	7.7
10	4	7.6
11	4	7.8
12	4	7.7
13	4	8.2
14	4	7.7
15	4	7.5
16	4	7.0
17	4	8.2
18	4	7.8
19	3	6.5
20	4	7.1
21	4	8.0
22	3	6.7
X	4	8.0
Y	4	8.1

FIG. 76A-B

a


target 2
target 4
target 3
target 5
target 7
target 6

b

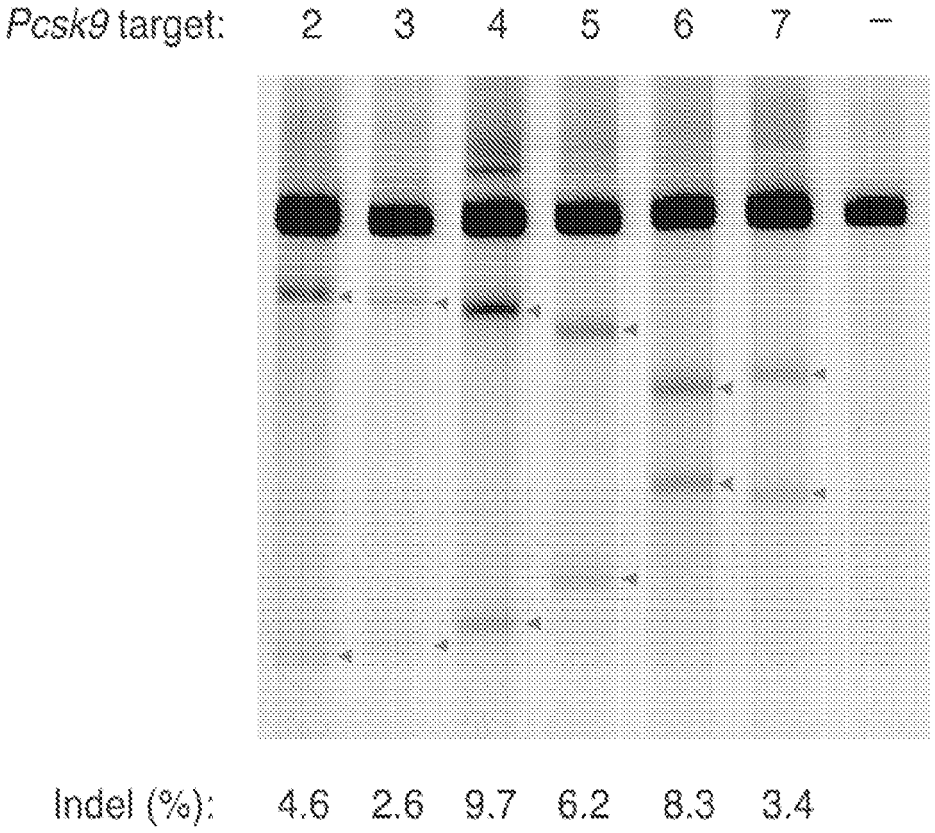
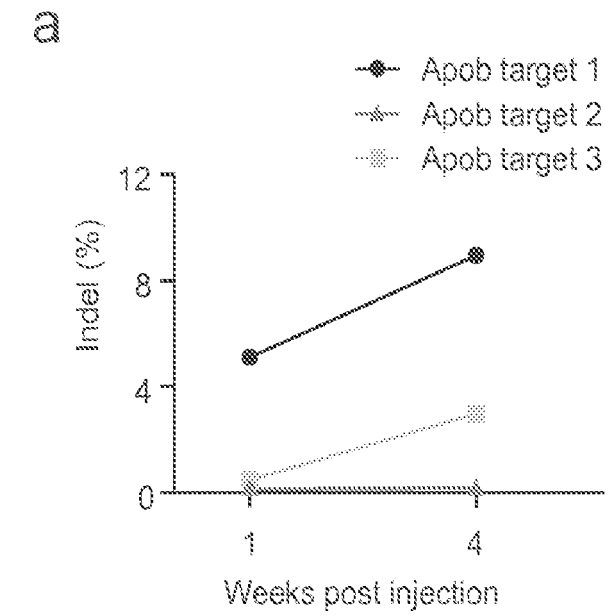
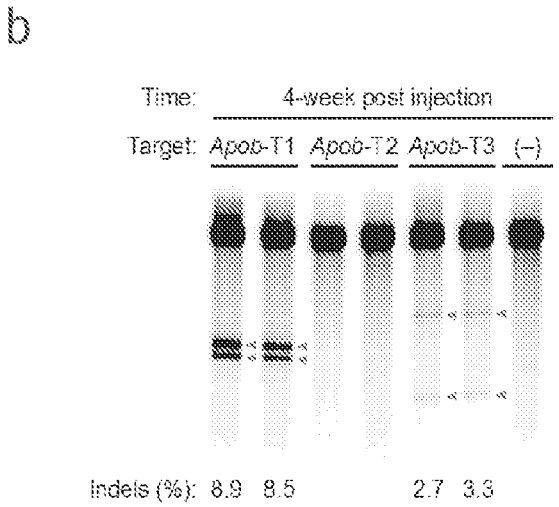

Pcsk9 target: 2 3 4 5 6 7 –
Indel (%): 4.6 2.6 9.7 6.2 8.3 3.4

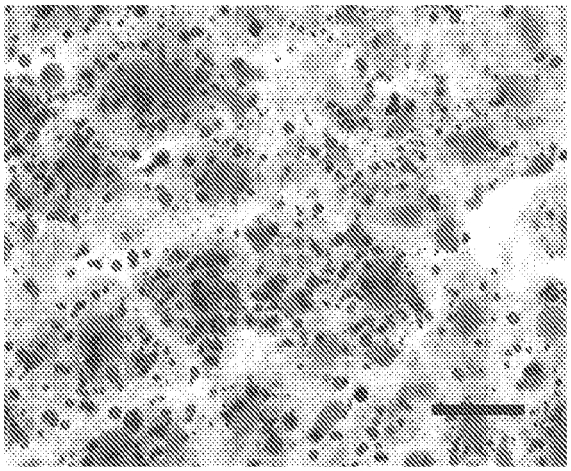
FIG. 77A-B



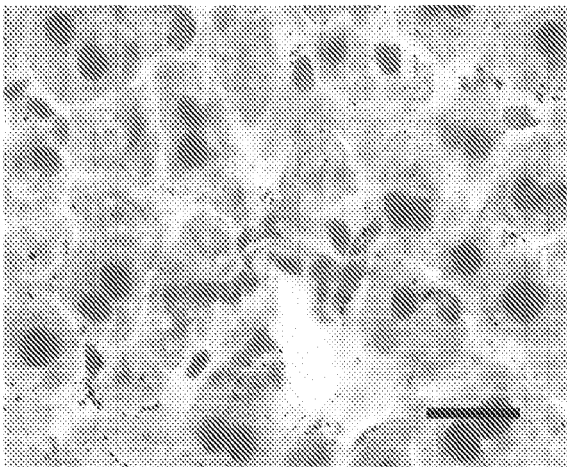


ApoB-T1 = Target 1; ApoB-T2 = Target 2, etc.

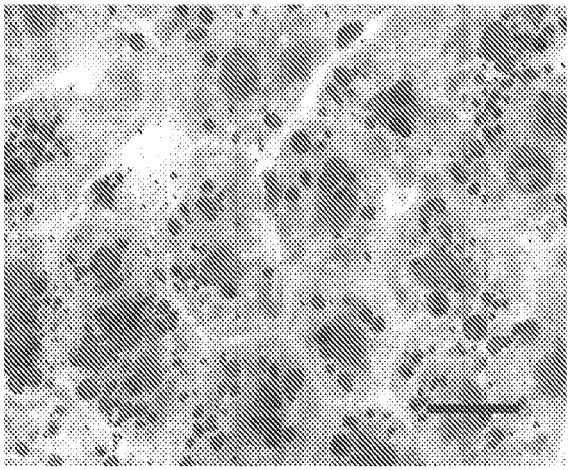
FIG. 78



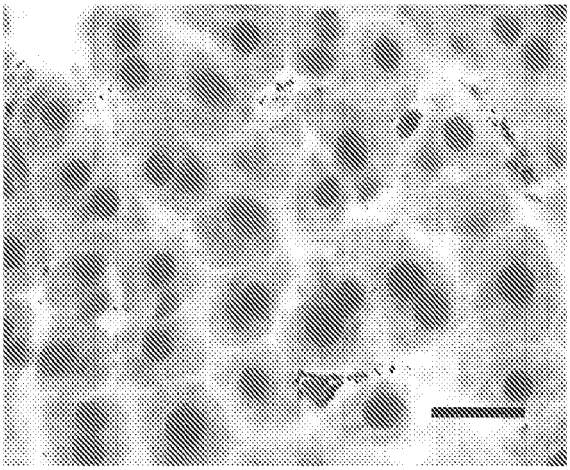
TBG-SaCas9-U6-sgRNA(Apob-T1)
4wk post injection



TBG-SaCas9-U6-sgRNA(Apob-T2)
4wk post injection

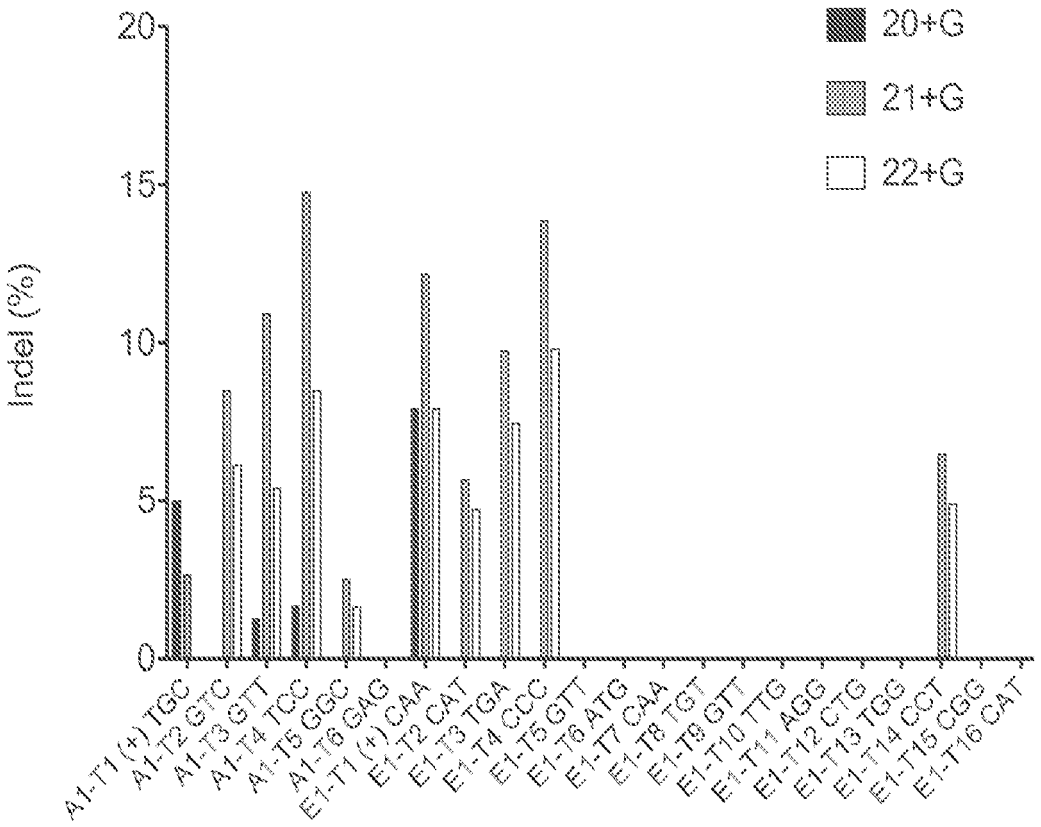


TBG-SaCas9-U6-sgRNA(Apob-T3)
4wk post injection



Wildtype (saline control)
4wk post injection

FIG. 79



AAVS1 & EMX1 targets
with first 3 base of the spacer (position 22,21,20)

FIG. 80

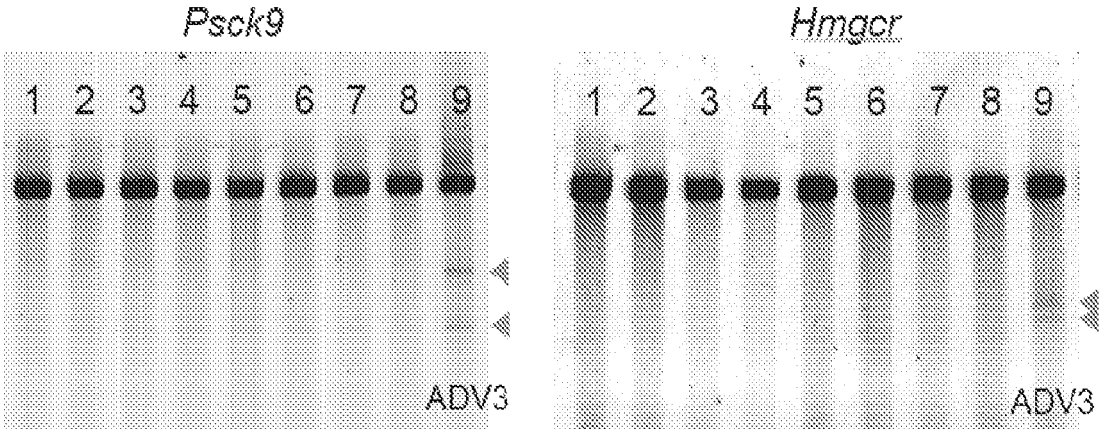


FIG. 81

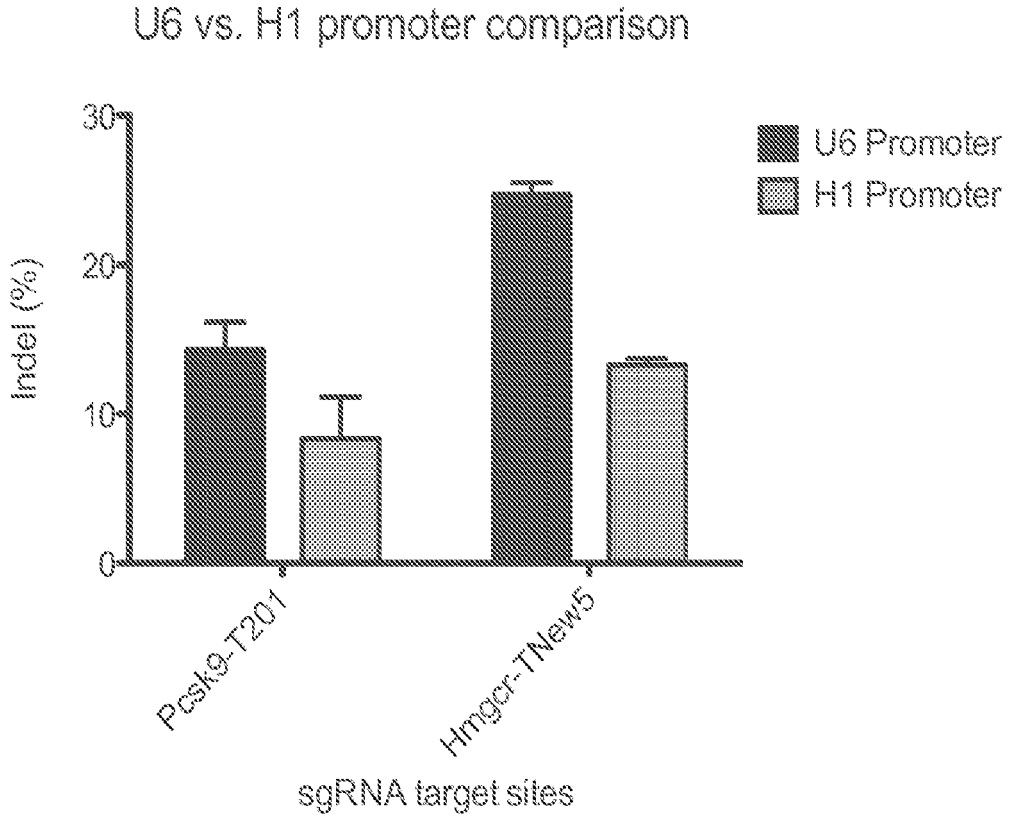

U6 vs. H1 promoter comparison
U6 Promoter
H1 Promoter
Indel (%)
30
20
10
0
Pcsk9-T201
Hmgcr-TNew5
sgRNA target sites

FIG. 82

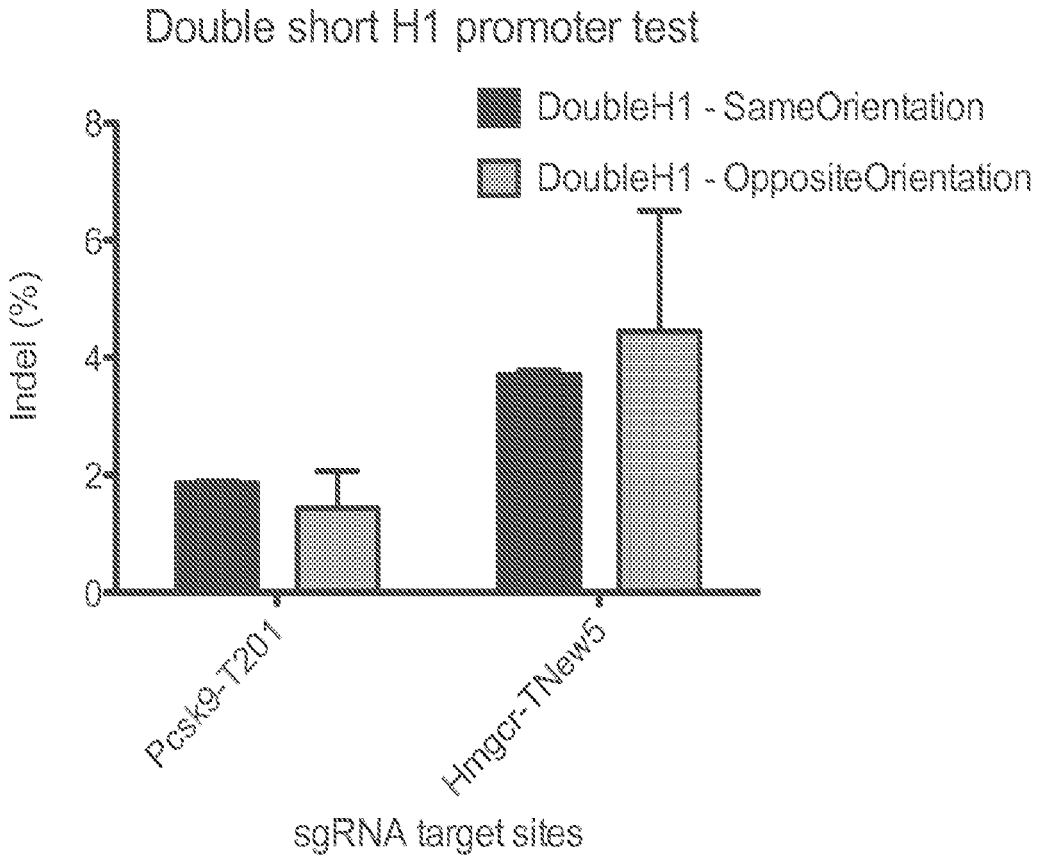

Double short H1 promoter test
DoubleH1 - SameOrientation
DoubleH1 - OppositeOrientation
Indel (%)
0
2
4
6
8
Pcsk9-T201
Hmgcr-TNew5
sgRNA target sites

FIG. 83

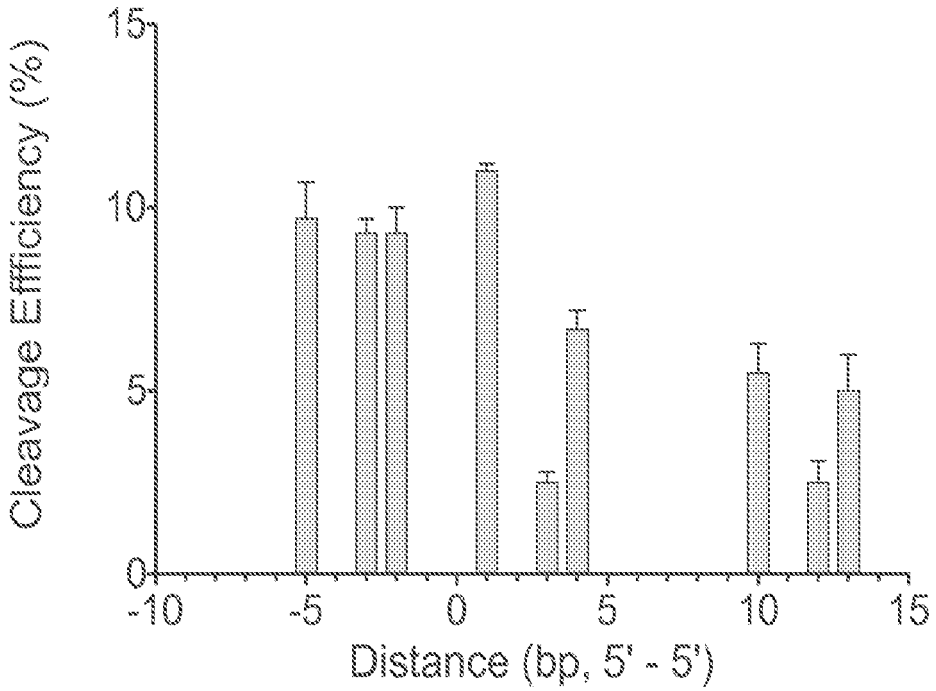


FIG. 84

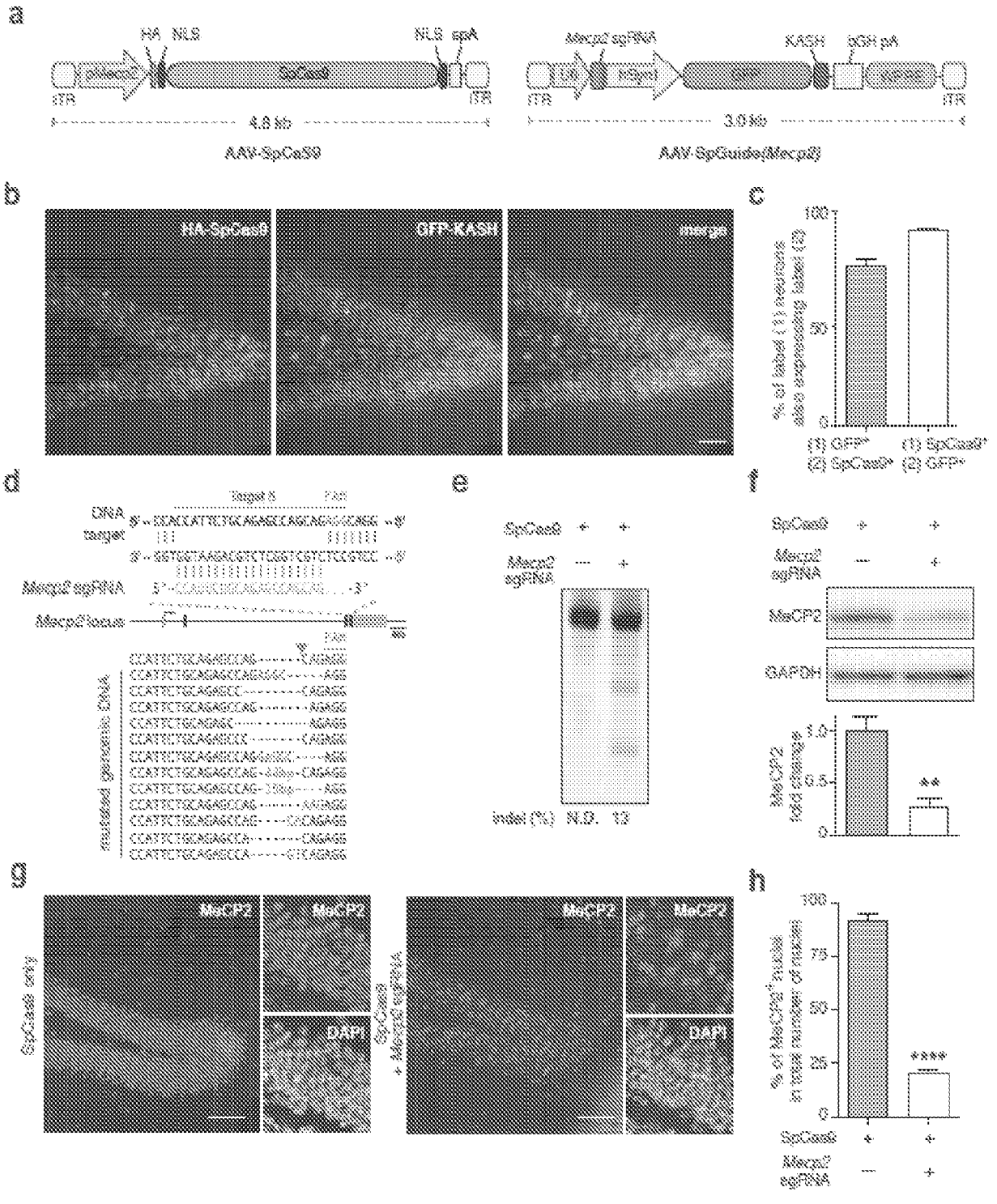

a
HA NLS
NLS spA
ITR
4.8 kb
AAV-SpCas9
Mecp2 sgRNA
KASH
bGH pA
3.0 kb
AAV-SpGuide(Mecp2)
b
HA-SpCas9
GFP-KASH
merge
c
(1) GFP+
(2) SpCas9+
(1) SpCas9+
(2) GFP+
d
DNA target
Mecp2 sgRNA
Mecp2 locus
Exon
wt
mutated genomic DNA
e
SpCas9
Mecp2 sgRNA
indel (%) N.D. 13
f
SpCas9
Mecp2 sgRNA
MeCP2
GAPDH
MeCP2 fold change
**
g
SpCas9 only
SpCas9 + Mecp2 sgRNA
MeCP2
DAPI
h
SpCas9
Mecp2 sgRNA
****

FIG. 85

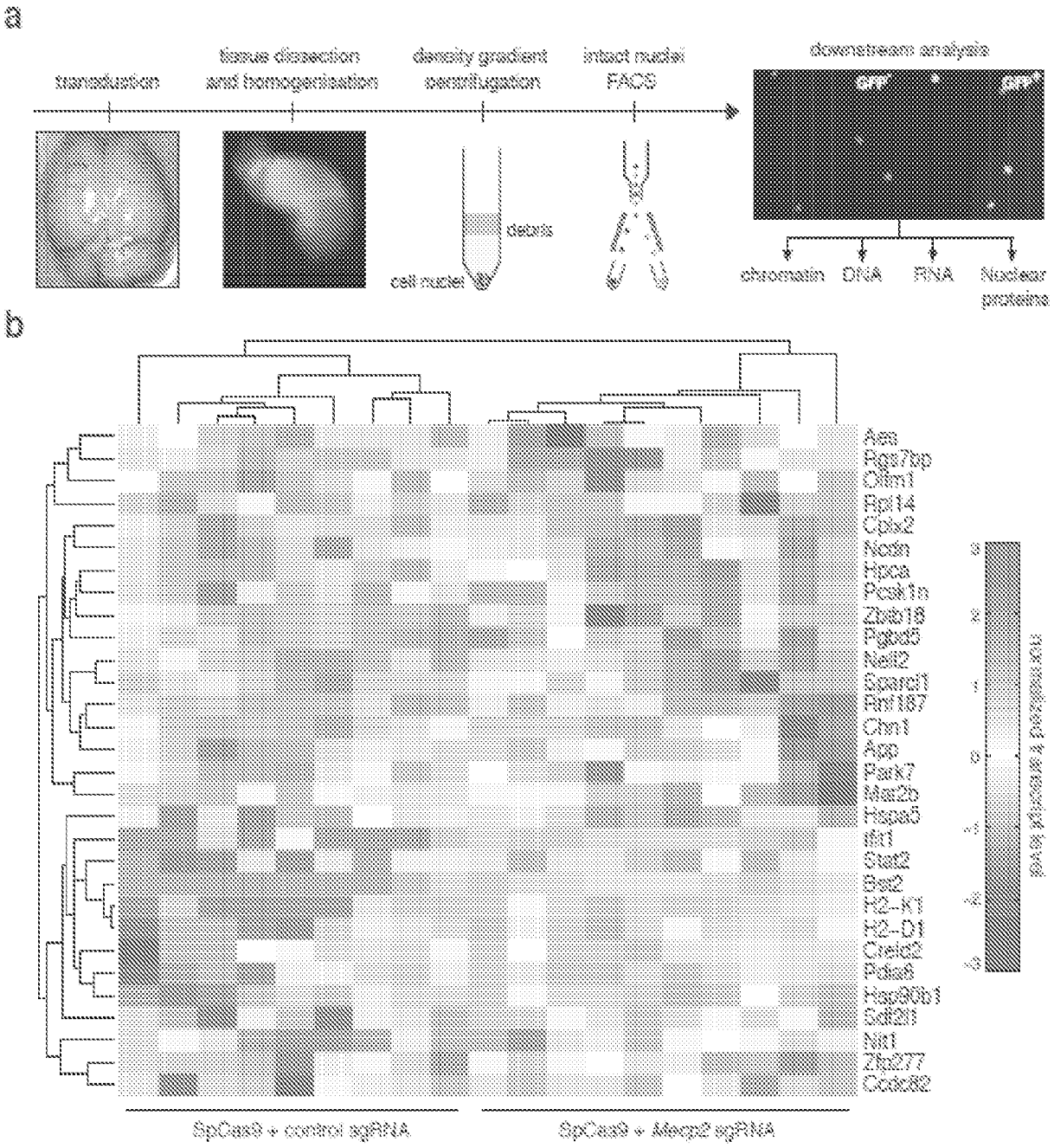

a
transduction
tissue dissection and homogenization
density gradient centrifugation
intact nuclei FACS
downstream analysis
DAPI
DAPI
debris
cell nuclei
chromatin
DNA
RNA
Nuclear proteins
b
Aes
Rps7bp
Olfm1
Rpl14
Cdx2
Ncdn
Hpca
Pcsk1n
Zbtb18
Pgbd5
Neil2
Sparcl1
Rnf187
Chn1
Ass
Park7
Mat2b
Hspa5
Ifit1
Stat2
Bst2
H2-K1
H2-D1
Ctsl62
Pdia6
Hsc90b1
Sdf2l1
Nit1
Zfp277
Ccdc82
SpCas9 + control sgRNA
SpCas9 + Mecp2 sgRNA
normalized expression level

FIG. 86

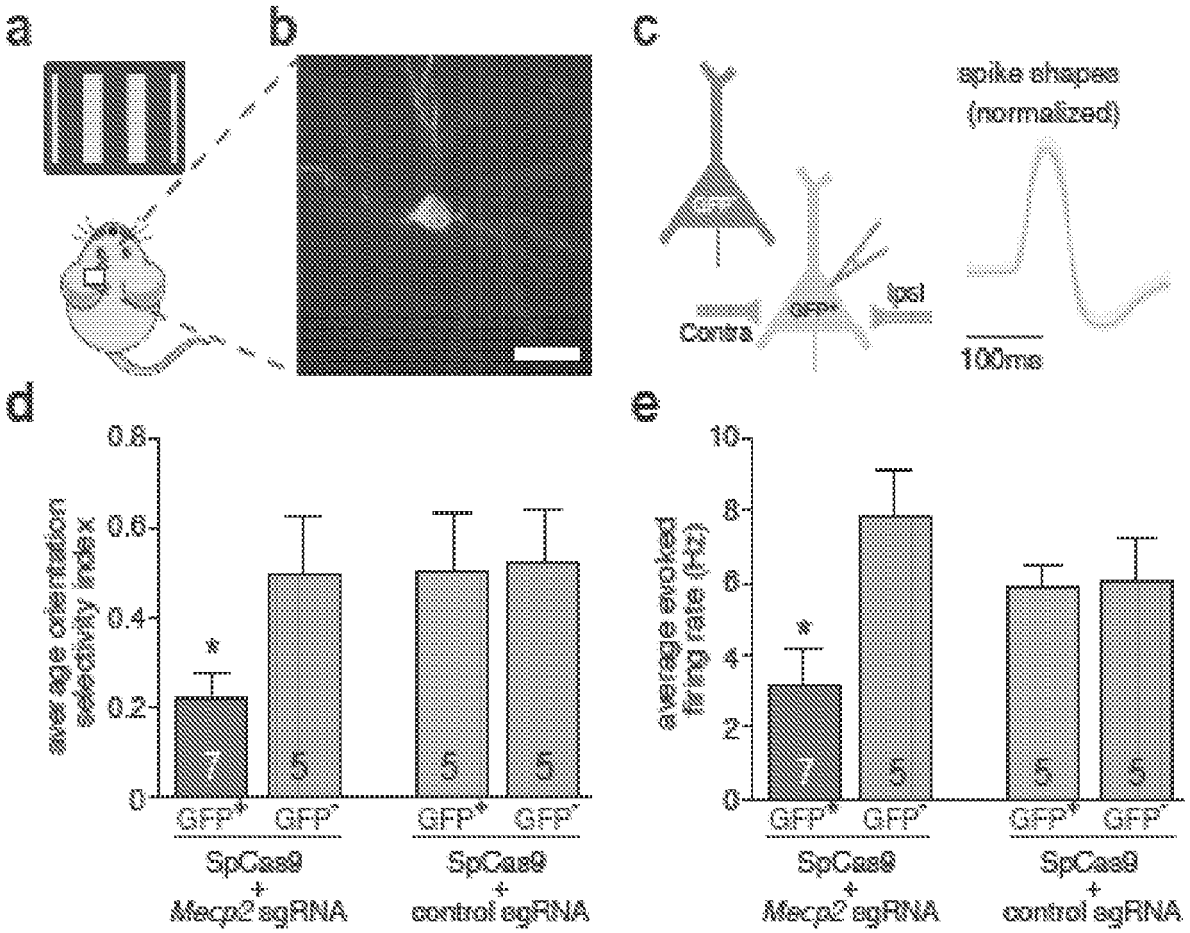

a
b
c
spike shapes
(normalized)
Contra
Ipsi
100ms
d
e
average evoked firing rate (Hz)
GFP+
GFP-
SpCas9
+
Mecp2 sgRNA
SpCas9
+
control sgRNA

FIG. 87

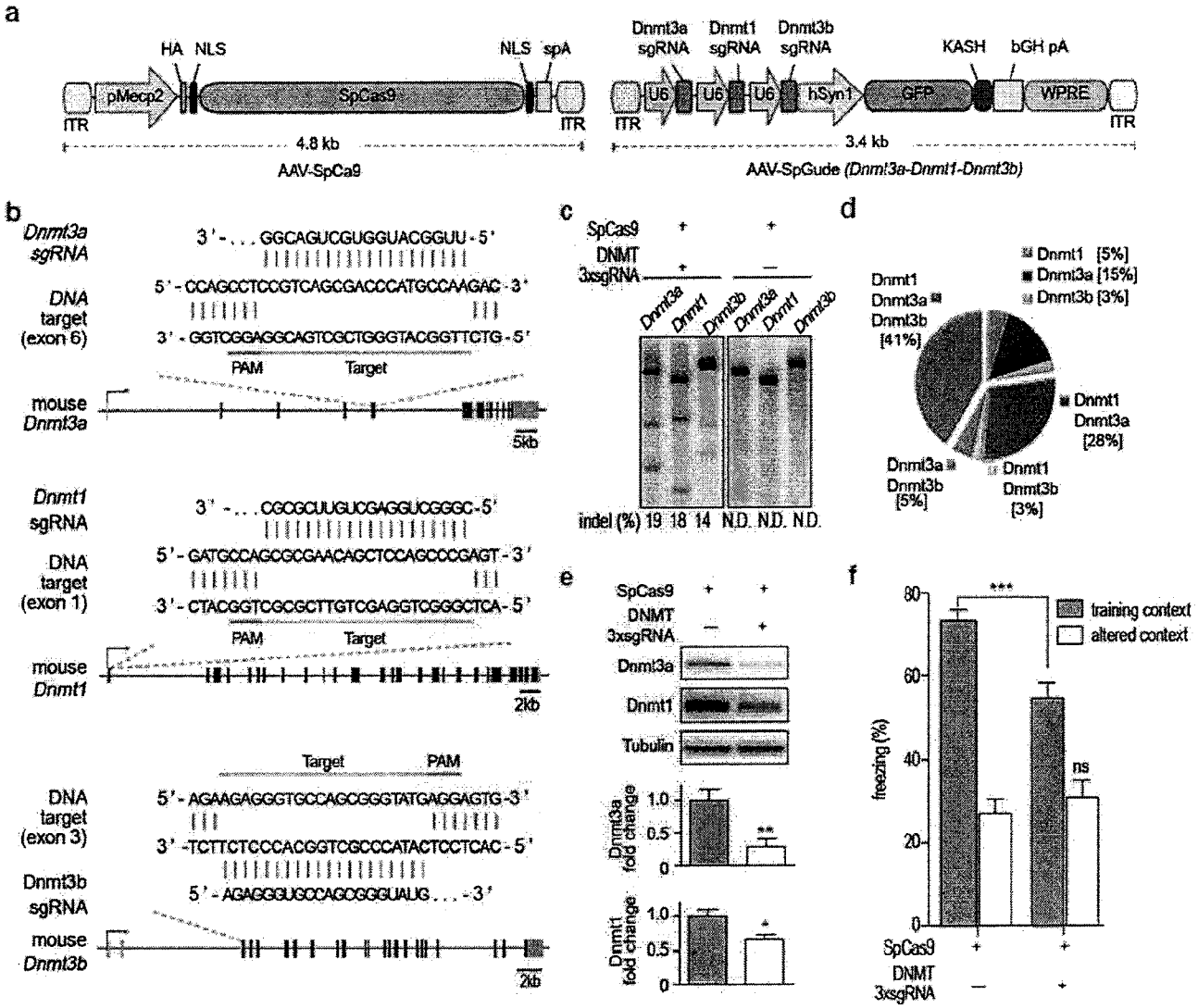

a
HA NLS
NLS spA
pMecp2
SpCas9
ITR
ITR
4.8 kb
AAV-SpCa9
Dnmt3a sgRNA
Dnmt1 sgRNA
Dnmt3b sgRNA
KASH
bGH pA
U6
U6
U6
hSyn1
GFP
WPRE
ITR
ITR
3.4 kb
AAV-SpGuide (Dnmt3a-Dnmt1-Dnmt3b)
b
Dnmt3a sgRNA
3'-...GGCAGUCGUGGUACGGUU-5'
DNA target (exon 6)
5'-CCAGCCTCCGTCAGCGACCCATGCCAAGAC-3'
3'-GGTCGGAGGCAGTCGCTGGGTACGGTTCTG-5'
PAM
Target
mouse Dnmt3a
5kb
Dnmt1 sgRNA
3'-...CGCGCUUGUCGAGGUCGGGC-5'
DNA target (exon 1)
5'-GATGCCAGCGCGAACAGCTCCAGCCCGAGT-3'
3'-CTACGGTCGCGCTTGTCGAGGTCGGGCTCA-5'
PAM
Target
mouse Dnmt1
2kb
Target
PAM
DNA target (exon 3)
5'-AGAAGAGGGTGCCAGCGGGTATGAGGAGTG-3'
3'-TCTTCTCCCACGGTCGCCCATACTCCTCAC-5'
Dnmt3b sgRNA
5'-AGAGGGUGCCAGCGGGUAUG...-3'
mouse Dnmt3b
2kb
c
SpCas9
DNMT 3xsgRNA
Dnmt3a
Dnmt1
Dnmt3b
Dnmt3a
Dnmt1
Dnmt3b
indel (%) 19 18 14 N.D. N.D. N.D.
d
Dnmt1 [5%]
Dnmt3a [15%]
Dnmt3b [3%]
Dnmt1 Dnmt3a Dnmt3b [41%]
Dnmt1 Dnmt3a [28%]
Dnmt3a Dnmt3b [5%]
Dnmt1 Dnmt3b [3%]
e
SpCas9
DNMT 3xsgRNA
Dnmt3a
Dnmt1
Tubulin
Dnmt3a fold change
1.0
0.5
0
**
Dnmt1 fold change
1.0
0.5
0
*
f
***
training context
altered context
freezing (%)
80
60
40
20
0
ns
SpCas9
DNMT 3xsgRNA

FIG. 88

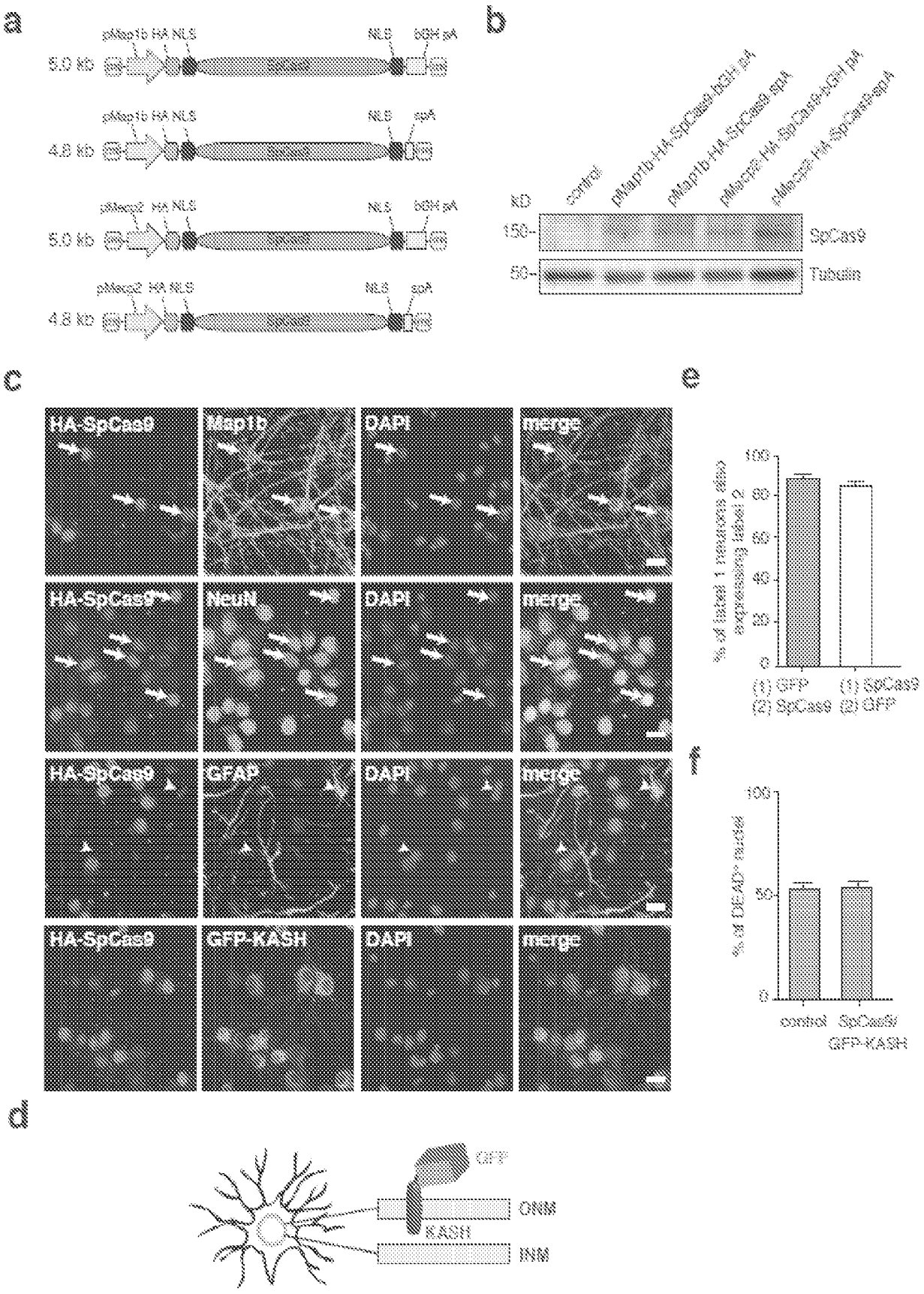

a
pMap1b HA NLS
NLS bGH pA
5.0 kb
pMap1b HA NLS
NLS spA
4.8 kb
pMecp2 HA NLS
NLS bGH pA
5.0 kb
pMecp2 HA NLS
NLS spA
4.8 kb
b
control
pMap1b-HA-SpCas9-bGH pA
pMap1b-HA-SpCas9 spA
pMecp2 HA-SpCas9-bGH pA
pMecp2 HA-SpCas9-spA
KD
150-
50-
SpCas9
Tubulin
c
HA-SpCas9
Map1b
DAPI
merge
HA-SpCas9
NeuN
DAPI
merge
HA-SpCas9
GFAP
DAPI
merge
HA-SpCas9
GFP-KASH
DAPI
merge
e
% of labeled 1 neurons also expressing label 2
100
80
60
40
20
0
(1) GFP
(2) SpCas9
(1) SpCas9
(2) GFP
f
% of DEAD* nuclei
100
50
0
control
SpCas9/ GFP-KASH
d
GFP
ONM
KASH
INM

FIG. 89

a

	Mecp2 target sequence	PAM
Target 1	CTGGGAGAGGGAGCCCCTCC	AGG
Target 2	AAAGGTGGGAGACACCTCCT	TGG
Target 3	TCCAACCTTCAGGCAAGGTG	GGG
Target 4	AGGAAGTCTGGCCGATCTGC	TGG
Target 5	CCATTCTGCAGAGCCAGCAG	AGG
Target 6	CTCTGAGGCCCTGGAGATCC	TGG

b

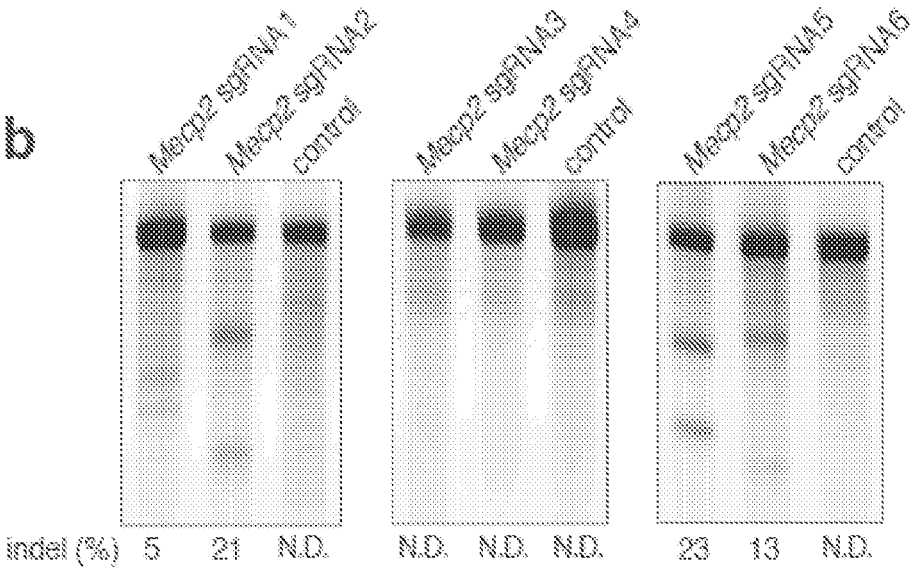


FIG. 90

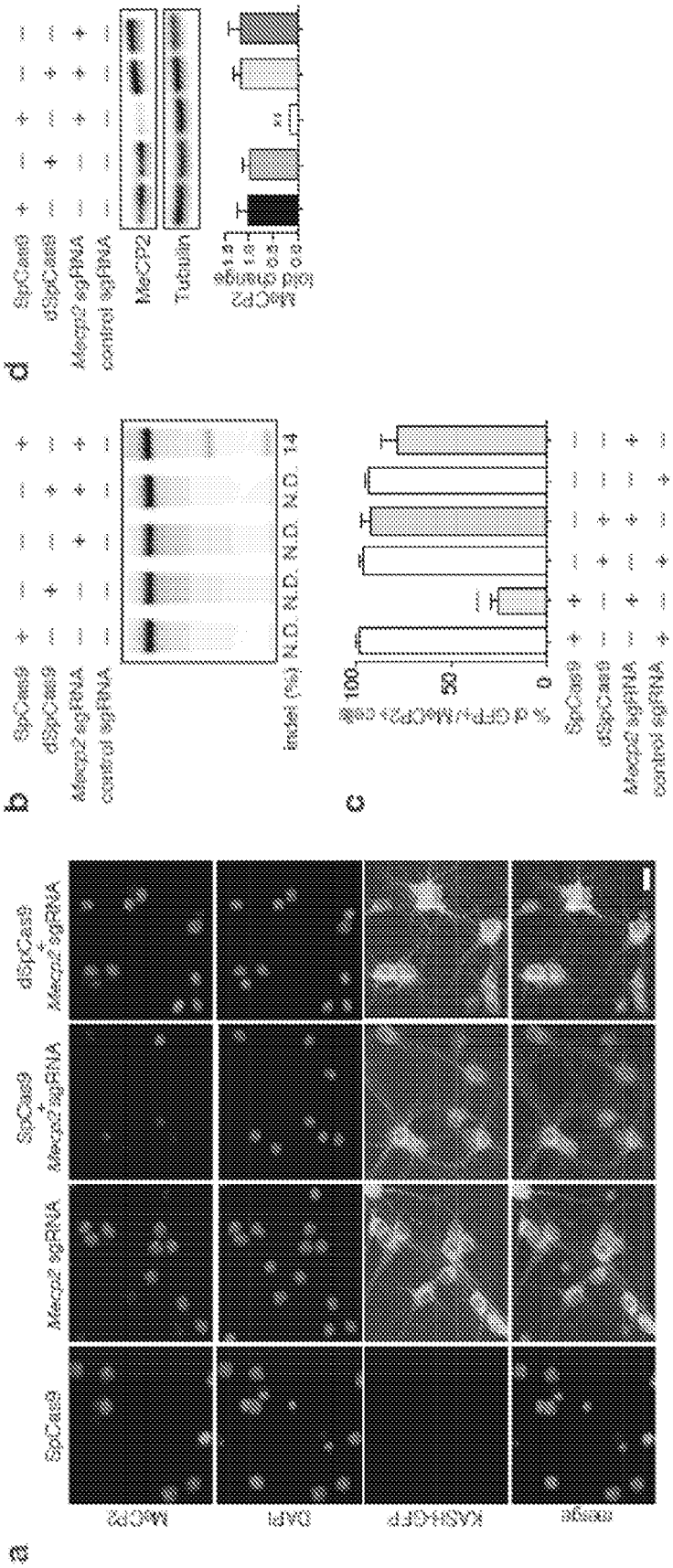

a
SpCas9
Mecp2 sgRNA
SpCas9 + Mecp2 sgRNA
dSpCas9 + Mecp2 sgRNA
MeCP2
DAPI
KASH-GFP
merge
b
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
Indel (%) N.D. N.D. N.D. N.D. 14
c
% of GFP+/MeCP2- cells
100
50
0
d
MeCP2
Tubulin
MeCP2 (%)
100
50
0

FIG. 91

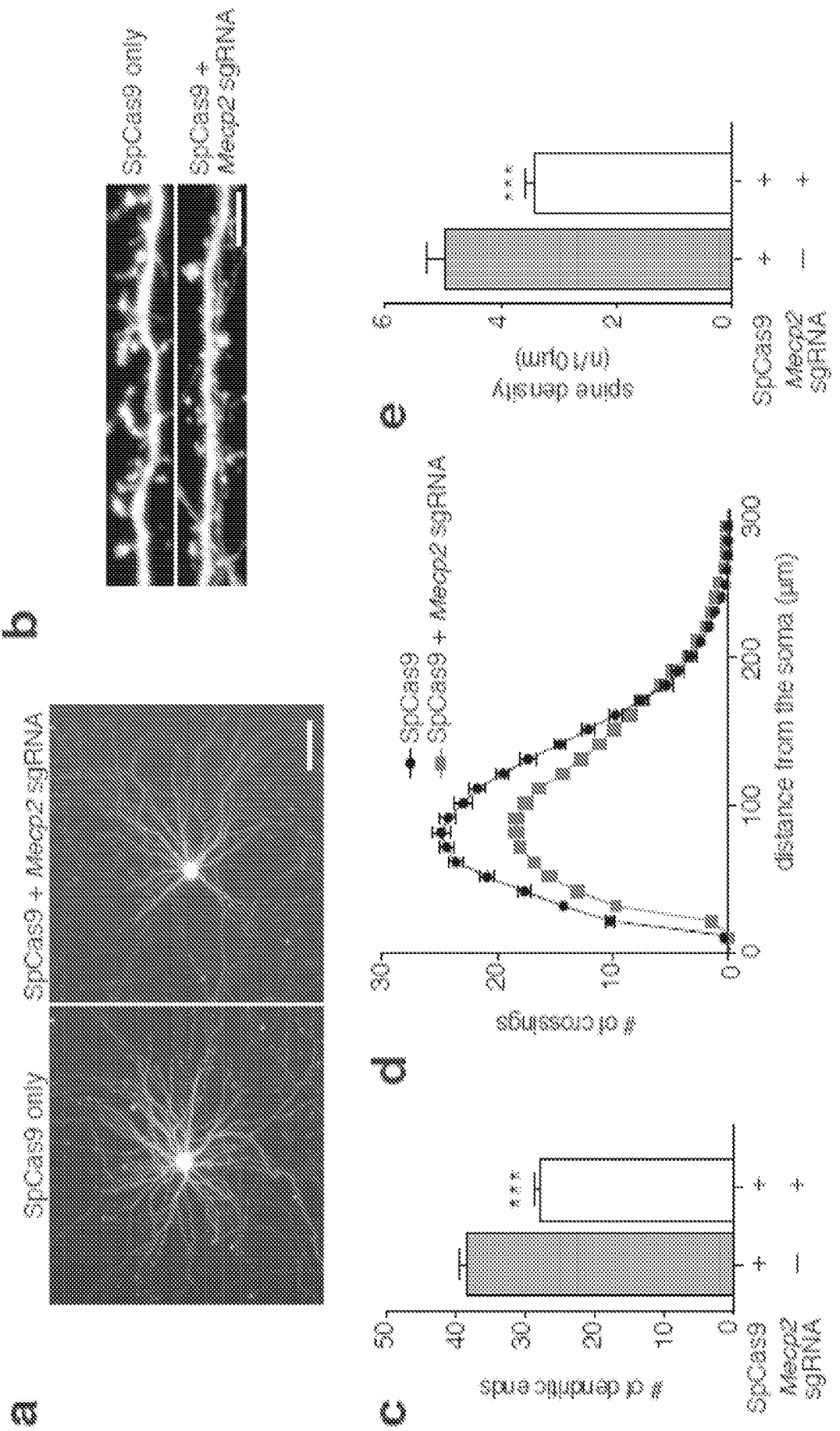

a
SpCas9 only
SpCas9 + Mecp2 sgRNA
b
SpCas9 only
SpCas9 + Mecp2 sgRNA
c
# of dendritic ends
SpCas9
Mecp2 sgRNA
d
# of crossings
distance from the soma (μm)
SpCas9
SpCas9 + Mecp2 sgRNA
e
spine density (n/1 0μm)
SpCas9
Mecp2 sgRNA

FIG. 92

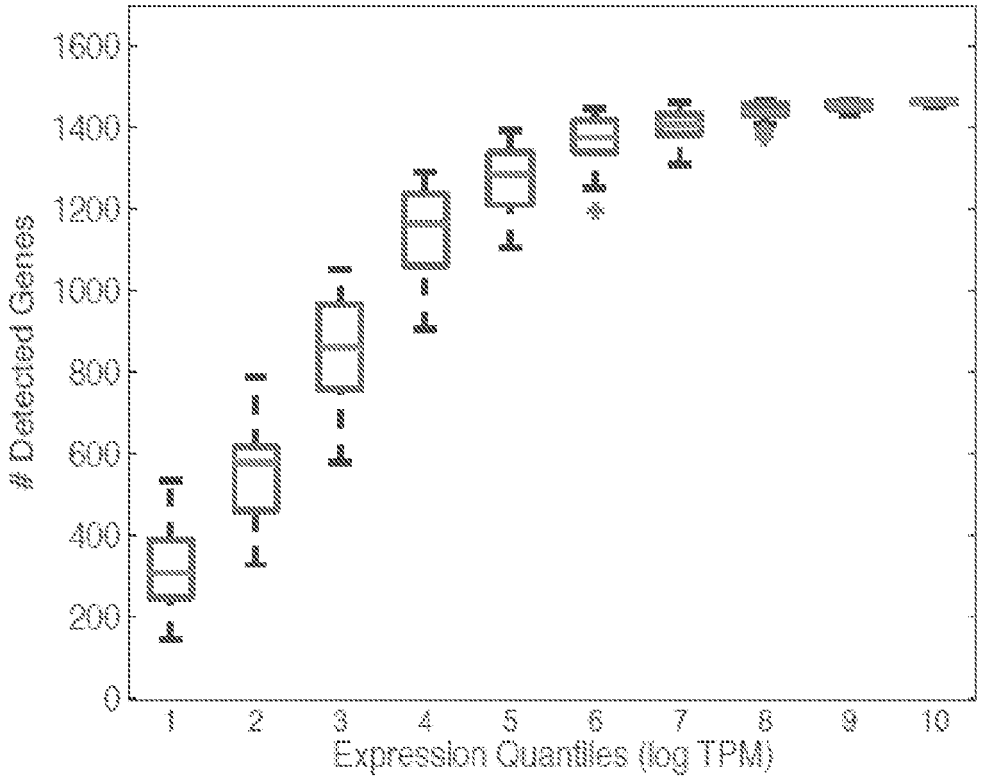

Detected Genes
0
200
400
600
800
1000
1200
1400
1600
1
2
3
4
5
6
7
8
9
10
Expression Quantiles (log TPM)

FIG. 93

a

target	target sequence	PAM
Dnmt3a	TTGGCATGGGTCGCTGACGG	AGG
Dnmt1	CGGGCTGGAGCTGTTCGCGC	TGG
Dnmt3b	AGAGGGTGCCAGCGGGTATG	AGG

b

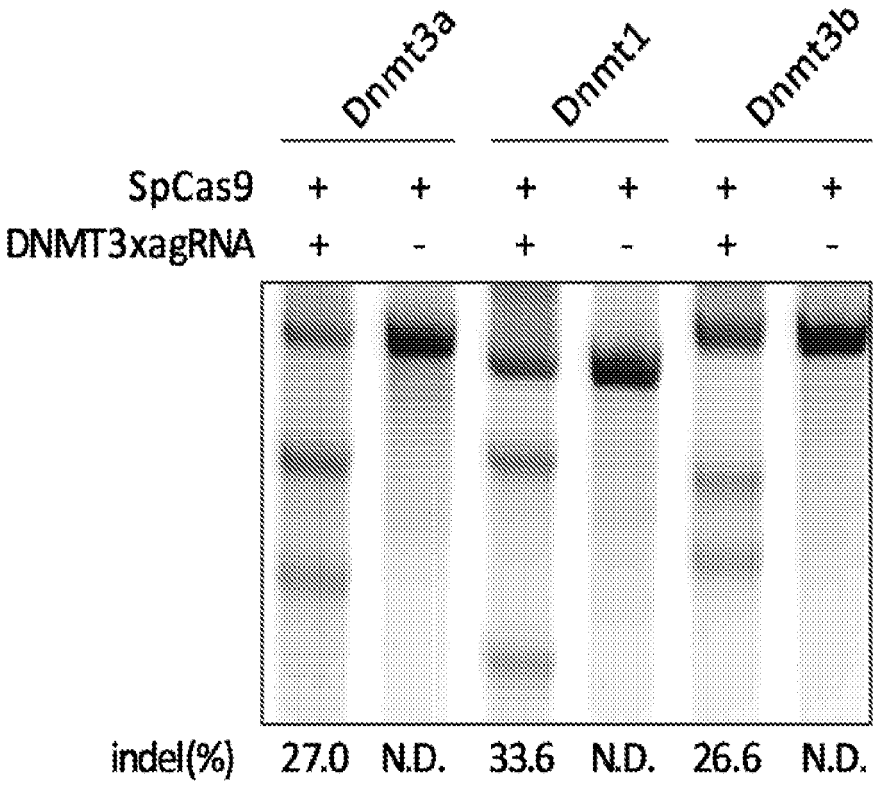


FIG. 94

a
Dnmt3a locus
PAM
CCTCCG--TCAGCGACCCATGCCAA
CCTCCG-ATCAGCGACCCATGCCAA
CCTCCGTGTCAGCGACCCATGCCAA
CCTCCG-TTCAGCGACCCATGCCAA
CCTCCG-CTCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG---CAGCGACCCATGCCAA
CCTC----TCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG------CGACCCATGCCAA
CCTC------AGCGACCCATGCCAA
C-------TCAGCGACCCATGCCAA
b
Dnmt1 locus
PAM
CCAGCG-CGAACAGCTCCAGCCCG
CCAGCGCCGAACAGCTCCAGCCCG
CCAGCGTCGAACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCG--G-ACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG-------AGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCGC---ACAGCTCCAGCCCG
CCAGCGC---------TCCAGCCCG
c
Dnmt3b locus
PAM
AGAGGGTGCCAGCGGG--TATGAGG
AGAGGGTGCCAGCGGG-TTATGAGG
AGAGGGTGCCAGCGGGTATATGAGG
AGAGGGTGCCAGCGGG-TAATGAGG
AGAGGGTGCCAGCGG--T-19bp-
AGAGGGTGCCAG------TATGAGG
AGAGGGTGCCAGCGGG--T--GAGG
AGAGGGTGCCAGCG----------AGG
AGAGGGTGCCAGCGGG-----GAGG

FIG. 95

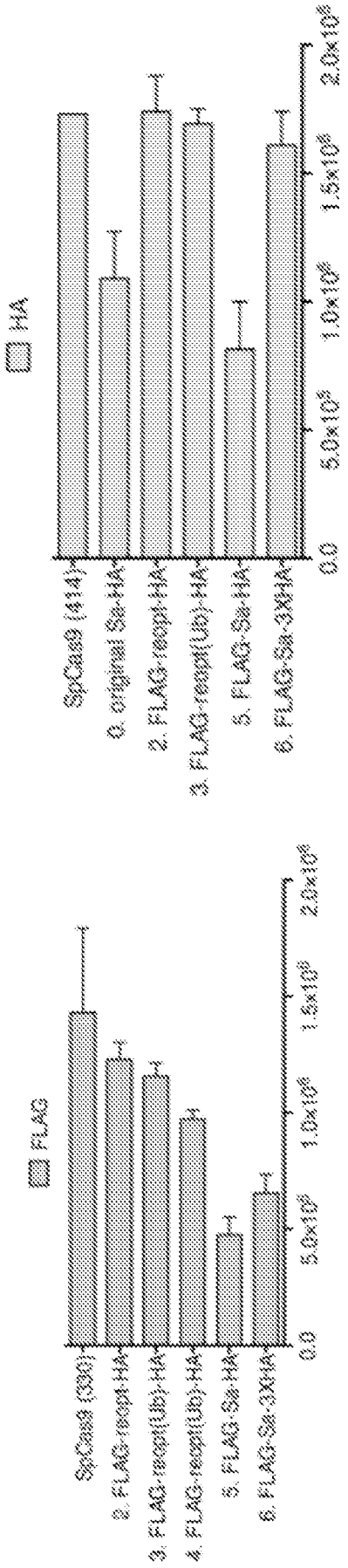

HA
SpCas9 (4114)
0. original Sa-HA
2. FLAG-reopt-HA
3. FLAG-reopt(Ub)-HA
5. FLAG-Sa-HA
6. FLAG-Sa-3XHA
0.0
5.0x10^5
1.0x10^6
1.5x10^6
2.0x10^6
FLAG
SpCas9 (330)
2. FLAG-reopt-HA
3. FLAG-reopt(Ub)-HA
4. FLAG-reopt(Ub)-HA
5. FLAG-Sa-HA
6. FLAG-Sa-3XHA
0.0
5.0x10^5
1.0x10^6
1.5x10^6
2.0x10^6

FIG. 96

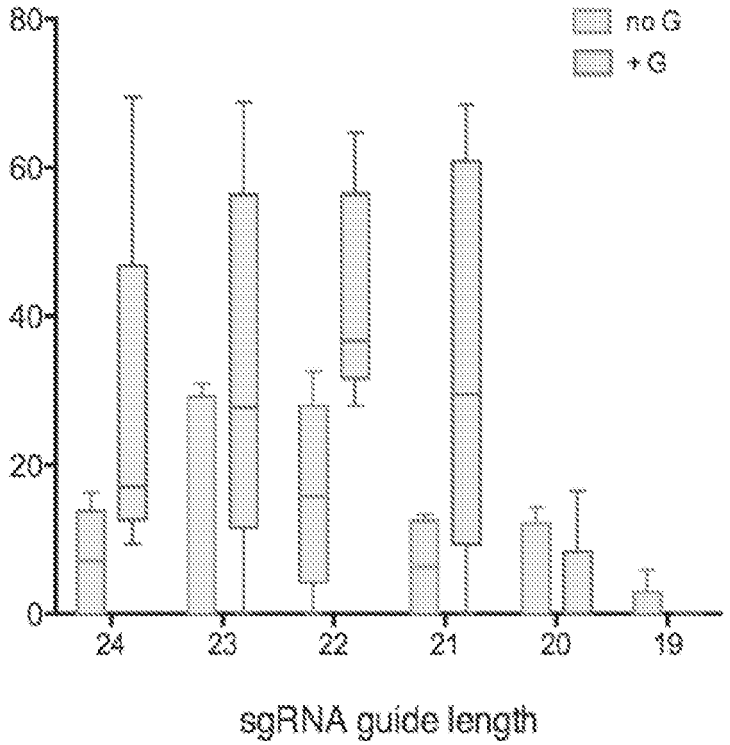
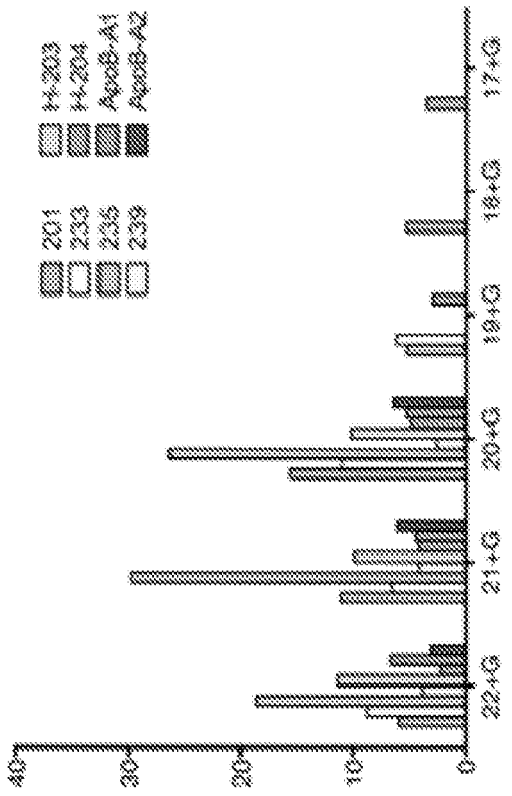


FIG. 97


H-303
H-204
Apo8-A1
Apo8-A2
201
233
235
239
40
30
20
10
0
22+G
21+G
20+G
19+G
18+G
17+G

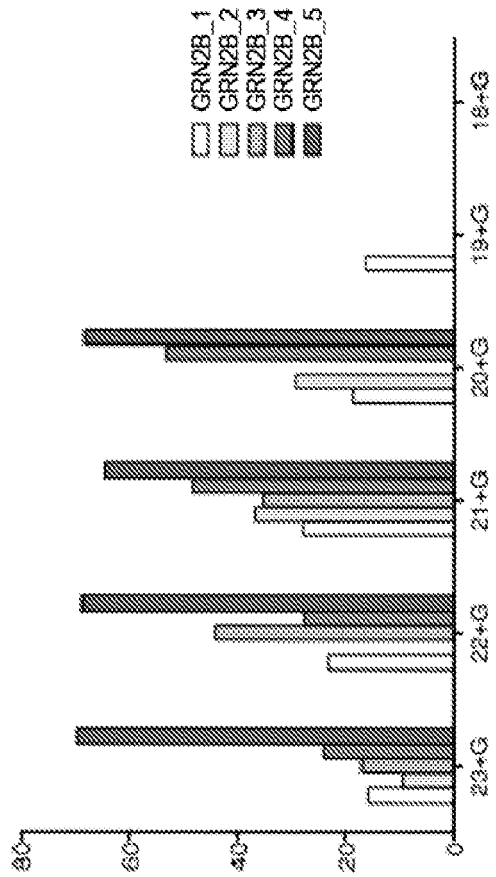

GRN2B_1
GRN2B_2
GRN2B_3
GRN2B_4
GRN2B_5
80
60
40
20
0
23+G
22+G
21+G
20+G
19+G
18+G

FIG. 98